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The acute and sub-acute toxicity of C_{60}/PVP complex *in vivo*

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Abstract. The detailed study of acute and sub-acute toxicity of the complex polyvinylpyrrolidon (PVP 20 kDa)-wrapped fullerene C_{60} after intraperitoneal (ip) administration was carried out on adult male Wistar rats. The LD_{50} value of C_{60}/PVP complex was found to be 7, 8 g/kg. In sub-acute study which lasted for 30 days the rats were exposed to daily administration of the complex in the doses of 350 or 700 mg/kg. All animals survived during the study and had no significant changes in clinical signs, organ weight, hematological and biochemical parameters of blood. The electrophysiological properties of myocardium and the excretory function of kidneys remained normal. Histological analysis of liver, kidney and spleen at the end of the study also did not demonstrate toxic alterations. It was thus established that intraperitoneal administration of complex C_{60}/PVP has no toxic effect. These results suggest that C_{60}/PVP has no acute and sub-acute toxicity and is a perspective substance for potential application in biology and medicine.

Keywords: fullerene C_{60}; polyvinylpyrrolidone; complex; toxicity

1. Introduction

Fullerene C_{60} (C_{60}) is the most studied carbon nanostructure and a single molecular form of carbon having well-defined unique physical and chemical properties. Molecule C_{60} having a high molecular weight is small in size - diameter spherical C_{60} MM 720 daltons approximately equals 0.7 nm (Anish *et al*. 2004, Guldi *et al*. 2006). Small size, high surface area and high reactivity of C_{60} make it interesting and useful for biological and medical applications. It was found that in biological systems C_{60} and its derivatives demonstrate three types of activity - antioxidant («radical sponge»), oxidant and membranotropic (Piotrovsky *et al*. 2006, Xiao and Wiesner 2012, Andrievsky *et al*. 1995, Long *et al*. 1995, Dugan *et al*. 1992, 1997, 2001). C_{60} and its derivatives have been characterized as novel powerful anti-oxidants, and are believed to reduce intracellular ROS and prevent oxidative cell injury. It was reported that fullerene C_{60} has a great affinity to free radicals (Krustic 1991). And its antioxidant ability is several hundred times higher than that of other antioxidants and it can effectively protect against all damaging forms of ROS: hydrogen peroxide, hydroxyl radical, and superoxide **(**Dugan *et al*. 2001, Kolosnja *et al*. 2007). At present

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time, the fullerenes production and usage in the market is limited, but it is expected to grow significantly in the nearest future (Aschberger *et al*. 2010, Murayama *et al*. 2005).

The possibility of using C_{60} in biology and medicine has been discussed since its discovery and to the present day (Piotrovsky *et al*. 2006, Bakry *et al*. 2007, Anilkumar *et al*. 2011, Takahashi *et al*. 2012, Liu *et al*. 2014). In biological studies it was used in different forms (Gharbi *et al*. 2005, Piotrovsky *et al*. 2006, 2011, Yamakoshi *et al*. 1994, Brant *et al*. 2006, Baati *et al*. 2012). Data clearly shows that pristine C_{60} has no acute or sub-acute toxicity in a large variety of living organisms, however, some C_{60} derivatives can be highly toxic (Kolosnjaj *et al*. 2007). It is known that the toxicity of water-soluble fullerenes is a function of surface derivatisation of C_{60} molecule and depends on the form of its administration. Therefore, considering the potential medical application of C_{60} it is necessary to study not only the biological effects but also its toxicity.

The investigation of biological effects of C_{60} is complicated by its low solubility in water. (~1.11x10-11 M, log Pow 6.67) (Jafvert and Kulkarni 2008). Therefore pristine fullerene C_{60} can be used as suspensions, inclusion complexes with organic polymers or as solution in natural oil for studying the biological properties of the C_{60} (Piotrovsky *et al*. 2006, 2011, Gharbi *et al*. 2005, Mori *et al*. 2006, Yamakoshi *et al*. 1994, Baati *et al*. 2012, Hu *et al*. 2012) etc.

The first study reporting the *in vivo* effect of intraperitoneal (ip) administration of C_{60} was carried out in 1996 (Moussa *et al.* 1996) and up to date, the toxicity of C_{60} and its derivatives has been studied by many groups of researchers (Nielsen *et al*. 2008, Johnston *et al*. 2010, Aschberger *et al*. 2010, Aoshima *et al*. 2009, 2010, Xiao *et al*. 2012, Yamashita *et al*. 2013). A few researches have addressed the studying of the toxicity of C_{60} or its derivatives at ip administration to mammal (Park *et al*. 2010,) and the safety of C_{60} oral administration *in vivo* (Gharbi *et al*. 2005, Mori *et al*. 2006, Nielsen *et al*. 2008, Baati *et al*. 2012, Takahashi *et al*. 2012, Yamashita *et al*. 2013). Mechanisms of biological action of the pristine C_{60}, C_{60}/PVP complex and other C_{60} derivatives are ***can be*** different, because on one side the biological effects of various fullerene preparations depend on the aggregation state of C_{60} molecule (Piotrovsky 2008). On the other side, it is impossible to compare the bioactivity of pristine and substituted fullerene, as they are different substances (Piotrovsky 2006). Therefore it is necessary to study biological effects of pristine fullerene C_{60} ***, its*** inclusion complexes with organic polymers and each of its individual derivatives at various ways of administration.

Biological effects of C_{60} derivatives depend on the degree of aggregation of its molecules. Even small degree of aggregation can cause conformational shifts in molecular structure which alter the biological property of C_{60} derivatives and when studying the toxicity of fullerene it is better to use low-aggregated derivatives.

The complex C_{60}/PVP was described for the first time by Yamakoshi (Yamakoshi *et al.* 1994). The mechanism of the complex formation was studied by Krakovyak (Krakovyak *et al*. 2006). In the complex C_{60}/PVP (PVP mm 12000-20000) fullerene molecules are in the low aggregation state what is confirmed by UV-spectrum, in which the position of the adsorption maximum at 333-334 nm can be considered as the indicator of low aggregation (Bensasson *et al*. 1994). Such state of fullerene molecules allows them to penetrate through the biological membrane and therefore fullerene has the membranotropic activity. Its antiviral effect confirms this property (Piotrovsky *et al*. 2004).

Today C_{60} and C_{60}/PVP complex are used in some applications in medicine and cosmetics as skin caring products and skin whitening agents (Kato *et al*. 2009, 2010, Inui *et al*. 2011, Lens *et al*. 2009, Takada *et al*. 2006).

Study of the safety of C_{60}/PVP complex is a key area in the development of its application not only in cosmetics, but also in nanomedicine. For several years our group has been studying the different aspects of biological activity of C_{60}/PVP complex: the impact on some classes of viruses (Piotrovsky *et*

al. 2004, 2005, 2008, 2013), the prevention of the disturbance of long-term memory induced by cycloheximide (Podolski *et al*. 2002, 2004) etc. Our preliminary data of C_{60}/PVP complex toxicity demonstrated the lack of negative effects of its ip administration on some blood biochemical and morphological indices in rats (Popov *et al*. 2008). The objective of the present study is the more detailed investigation of sub-acute toxicity of C_{60}/PVP complex after ip injection in rats.

2. Materials and methods

C_{60} (99.5%) was purchased from NeoTechProduct (Saint Petersburg, Russia). Polyvinylpirrolidone (PVP, Povidone). The UV- vis measurements spectra were conducted on Beckman Coulter DU 800 spectroscopy system (USA) at room temperature, solvents - water and mixture of ethanol: toluene.

For toxicological studies adult male Wistar rats were obtained from Rappolovo nursery (Russia) and housed under standard conditions (constant temperature of 20-22℃, relative humidity of 50-65 % and 12-h light/dark cycle) and had free access to standard feed and water.

This investigation conforms to the Guide for the Care and Use of Laboratory Animals in Russian Federation.

2.1 Preparation and analysis of C_{60}/PVP complex

C_{60}/PVP complex was prepared and analyzed according (Krakovjak *et al*. 2005, 2006). The quality of C_{60}/PVP complex was tested by UV-spectra: in aqueous solution the UV spectrum of the complex has two peaks at 260 nm and 332-334 nm and two minima of 242 nm and 316 nm. Dynamic light scattering study of the hydrodynamic diameters of C_{60}/PVP showed: the particle size of C_{60}/PVP in the distilled water was 127 nm, zeta- potential was -2.2mV (Sushko *et al***.** 1999). C_{60} concentration in the complex C_{60}/PVP was determined by "heterophase" method (Krakovjak *et al*. 2005, 2006) and was 0.5-0.6 %.

2.2 Acute toxicity of the C_{60}/PVP

To evaluate the acute toxicity of C_{60}/PVP after a single ip injection 32 Wistar rats (body weight 200-220 g) were used. After the period of quarantine the rats were assigned randomly to two experimental groups and treated with C_{60}/PVP or PVP in the doses 5.0; 6.25; 8.0; 10.0 g/kg (n=4 for each dose). Increase on the C_{60}/PVP dose over 10 g/kg, which corresponds to 40% concentration, was impossible, since viscosity prevents its ip introduction. The time of death and clinical signs of intoxication were monitored during 14 days after injection. The dose dependent toxic effects of tested substances and their LD_{50} were determined by the conventional Prozorovski method (Prozorovski and Prozorovskaia 1980).

2.3 Sub-acute toxicity of C_{60}/PVP

The study was carried out on 120 Wistar rats (body weight 230-240 g). The animals were divided into 5 groups of 24 rats in each group: I - control, II - PVP-350 mg/kg, III - C_{60}/PVP-350 mg/kg, IV - PVP-700 mg/kg, V - C_{60}/PVP-700 mg/kg. The water solutions of PVP and C_{60}/PVP were administrated ip daily for 30 days. The doses 350 and 700 mg/kg are 1/20 and 1/10 LD_{50} of PVP. The control animals were administered an equal volume of water.

The functional state of the experimental animals was evaluated on the 14 and 30 days after the beginning of drug administration. The general indices, biochemical and hematological parameters, urinary system, the electrophysiological activity of the heart, and morphological study of liver, kidney and spleen were fulfilled.

General indices included the general state of the animals, appearance, behavior, daily food and water intake, body weight (once a week), and weight of the internal organs at the end of the experiment. Clinical signs of intoxication were recorded once a day during the study period.

Hematological parameters were evaluated in the blood received from the tail vein. Blood samples were collected in a micro tube with EDTA. Analysis was performed using fully automated hematology analyzer DREW D3 (USA). The following parameters were studied: the total number of white blood cells (WBC), total medium leukocyte number (MID #), a percentage of medium leukocytes (MID%), total number of lymphocyte (LYM #), the number of lymphocytes as a percentage (LYM%), the total number of granulocytes (GRA #), the number of granulocytes in the percentage (GRA%), the total number of red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean content of hemoglobin (MCH), the mean concentration of hemoglobin in corpuscular (MCHC), erythrocyte volume coefficient of variation (RDV), erythrocyte sedimentation rate (ESR), the total number of platelets (PLT), the mean volume of platelets (MPV).

For *biochemical test* blood samples were centrifugated at 1600 rpm. In serum the activity of some blood marker enzymes and indicators of protein, carbohydrate and lipid metabolism were studied. The activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) were studied by Reitman-Frankel unificated method (Reitman *et al*. 1957) and alcaline phosphatase (ALP) by standart method (Bessey *et al*. 1946). Total cholesterol (TC), triglycerides (TG), cholesterol concentration of antiatherogenic high-density lipoprotein (HDL), total protein content (TP), albumin (A), and glucose were determined by standardized enzymatic methods on analyzer STAT FAX-3300 (USA). Kits "Vital Development Corporation" (Russia) were used. Globulin content (G) was calculated as the difference of TP and A. The ratio of albumin to globulin (A/G) was also calculated.

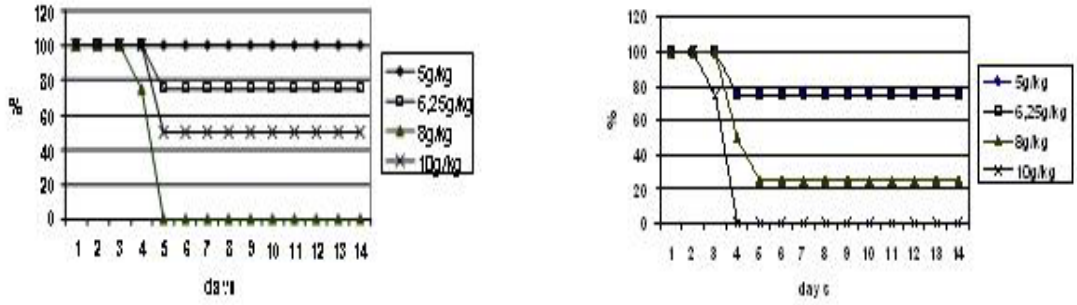
Bioelectric activity of the rat heart muscle was analyzed on ECG (electrocardiograph EKCHMP-N3051, Russia; speed 200 mm/s, gain 20 mm/mV). The duration of RR, P(Q)R, (Q)T intervals and (Q)RS complex were determined. T-wave amplitude was estimated for the control of myocardial ischemia. All measurements were performed in standard lead II.

The excretory function of kidneys. Hourly urine output (with a water load) and physiological characteristics of urine (specific gravity, pH, protein, sugar, ketone bodies) were determined. Urine test was performed on an automated portable device URILYUKS (Boehringer Mannheim GmbH, Germany) on a standardized test strips. Microscopic analysis of the sediment, including determination of the leucocytes, erythrocytes, bacteria, and triple phosphates was fulfilled. Blood urease was analyzed using urea phenol/hypochlorite method with kit "Vital Development Corporation" (Russia).

Morphological study of liver, kidney and spleen of animals treated with tested agents within 30 days was carried out. The material was fixed in 10% formalin. The histological structure of the organs was analyzed on semithin sections stained with hematoxylin and eosin.

2.4 Statistical analyses

Means were compared with one-way ANOVA. If ANOVA result indicated significant effect ($p \leq 0.05$), then post hoc comparisons were made. At that, if Levene`s test of homogeneity of variances was not significant ($p > 0.05$), i.e., the assumption of equal variances was not violated, then Bonferroni, Sidak and Tukey tests were used. If variances were not homogenous, then Dunnett`s 3T and Games-



(a) C_{60}/PVP (b) PVP

Fig. 1 Dynamics of the survival after ip administration of C_{60}/PVP and PVP

Howell tests were used. Means were considered to be different significantly at $p \leq 0.05$. SPSS was used for all statistical calculations.

3. Results

3.1 Acute toxicity of C_{60}/PVP

The survival of the rats after single ip C_{60}/PVP or PVP injection at the doses ranging from 5 to 10 g/kg allowed to establish the LD_{50} values. For C_{60}/PVP it was 7,9±1,0 g/kg and for PVP - 7,0±0,7 g/kg. The death of the animals receiving C_{60}/PVP complex happened on the 4th and 5th day, and in the group of animals treated with PVP - on 3d - 4th day (Fig. 1). After lethal doses injection the animals appeared flaccid, alternating with retardation. Neurotoxic effects of drugs were not noted.

The macroscopic study of the animals after euthanasia on the 14th day of the experiment did not show the presence of local irritant action of the C_{60}/PVP complex and PVP. Edema, hyperemia, and necrosis of skin and hypodermic fatty tissue in the place of drug injection were not observed. There were no visible changes of internal organs.

3.2 Sub-acute toxicity of C_{60}/PVP

3.2.1 General indices

Daily monitoring of the animals receiving PVP or C_{60}/PVP in doses of 350 and 700 mg/kg had not revealed any deviations in their state and behavior in comparison with control group. The difference in weight of internal organs in control and experimental groups was not statistically significant (p>0,05) (Table 1).

3.2.2 Hematological and biochemical parameters

The cellular composition of the peripheral blood on the 14 and 30 day of the experiment remained normal; therefore only data for 30th day are shown in Table 2.

The exposure to C_{60}/PVP and PVP did not lead to any significant changes in the activity of blood serum marker-enzymes. This suggests that long-term ip injections of C_{60}/PVP and PVP did not result in tissue lesion.

Table 1 C_{60}/PVP and PVP influence on daily food and water intake and weight of internal organs on the 30th day of experiment

Index	Group				
	Control	PVP, 350 mg/kg	C_{60}/PVP, 350 mg/kg	PVP, 700 mg/kg	C_{60}/PVP, 700 mg/kg
Food intake, g	21.4±0.3	21.3±0.5	16.8±0.7*#	19.8±0.4	18.1±0.5*
Water intake, ml	36.0±1.3	35.2±1.1	24.6±1.2*#	33.8±1.5	31.1±1.1*
Organ weight, g					
-heart	0.999±0.039	0.978±0.047	0.969±0.067	0.921±0.038	0.889±0.027
-lungs	1.651±0.120	1.421±0.076	1.760±0.228	1.668±0.183	1.530±0.066
-liver	7.561±0.307	8.129±0.271	7.620±0.203	7.862±0.369	8.132±0.239
-spleen	1.150±0.071	1.275±0.042	1.467±0.100	1.235±0.136	1.319±0.140
-kidneys	1.914±0.120	1.894±0.034	1.782±0.107	1.886±0.107	1.844±0.042
-adrenals	0.043±0.003	0.049±0.002	0.047±0.005	0.048±0.001	0.043±0.002
-testicles	3.294±0.159	3.417±0.204	3.121±0.103	3.130±0.165	3.393±0.146
-thymus	0.332±0.039	0.329±0.025	0.420±0.029	0.326±0.029	0.343±0.021

*- difference with control is statistically significant ($p< 0.05$); # - difference with PVP is statistically significant ($p<0.05$); values are means ± SEM

Table 2 Effect of C_{60}/PVP and PVP on the blood and serum indices

Parameter	Group				
	Control	PVP, 350 mg/kg	C_{60}/PVP, 350 mg/kg	PVP, 700 mg/kg	C_{60}/PVP, 700 mg/kg
Serum					
Glucose, mM/l	4.4±0.4	4.2±0.4	4.5±0.2	4.6±0.6	4.8±1.0
TG, mM/l	0.8±0.1	0.8±0.1	0.9±0.1	0.8±0.1	0.8±0.2
TCh, mM/l	1.2±0.1	1.8±0.6	2.0±0.3*	1.8±0.5	1.5±0.3
HDL-Ch, mM/l	0.6±0.1	0.6±0.2	0.5±0.04	0.6±0.1	0.6±0.04
TP, g/l	65.9±7.0	62.9±3.2	64.4±3.3	63.5±2.0	66.4±2.9
Albumin, g/l	33±1.1	32±1.4	33±1.0	34±3.1	38±2.0
Globulin, g/l	33±7.9	29±2.8	32±2.7	30±3.6	28±3.0
A/G	1.1±0.1	1.2±0.2	1.1±0.2	1.2±0.1	1.3±0.2
AST, mM/h·l	1.35±0.09	1.17±0.03	1.06±0.03	1.19±0.07	1.22±0.10
ALT, mM/h·l	1.24±0.02	1.11±0.08	0.91±0.08	0.94±0.08	1.02±0.13
ALP, nM/s.l	913.1±96.6	731.4±41.4	911.6±107.8	755.1±51.5	837.4±71.9
Urea, mM/l	8.3±0.6	8.5±0.4	9.4±0.3	9.0±0.5	9.8±0.7
Blood					
WBC, G/L	20,9±2,4	19,4±2,4	19,7±1,3	23,7±3,1	21,6±1,6
MID, G/L	2,5±0,4	1,6±0,2	1,7±0,2	2,4±0,6	1,9±0,1
MID,%	12,2±1,3	8,6±0,7	8,6±1,1	9,9±1,5	9,1±1,0
LYM, G/L	13,8±2,0	14,2±2,1	14,8±1,2	17,8±2,6	16,3±1,8
LYM, %	64,7±3,7	72,6±3,3	74,9±2,6	74,1±3,6	74,5±2,9

Table 2 Continued

GRA, G/L	4,6±0,6	3,6±0,6	3,3±0,4	3,6±0,4	3,4±0,2
GRA, %	23,1±2,8	18,9±2,7	16,6±1,9	16,0±2,6	16,4±1,9
RBC, T/L	8,07±0,16	7,74±0,16	8,26±0,16	8,43±0,25	8,12±0,29
HGB, g/l	151,5±2,0	145,7±2,5	143,8±1,9	150,7±2,2	153,6±1,7
HCT, L/L	0,374±0,006	0,382±0,006	0,378±0,006	0,394±0,008	0,406±0,007
MCV, fl	49,7±0,9	49,4±0,9	45,8±0,4	46,9±0,9	50,2±1,6
MCH, pg	18,8±0,3	18,9±0,3	17,4±0,2	17,9±0,4	19,0±0,6
MCHC, g/l	378,7±2,3	381,7±2,2	380,5±2,7	382,3±2,6	378,4±3,1
PLT, g/l	1020,5±77,3	833,7±29,4	865,0±16,5	937,2±67,0	850,2±50,1
MPV, fl	6,1±0,1	5,7±0,2	5,8±0,1	6,0±0,1	5,9±0,1
ESR, mm/h	1,3±0,2	1,5±0,2	1,5±0,2	1,2±0,2	1,0±0,0

Values are means ± SEM

Table 3 Effect of C_{60}/PVP and PVP on the electrophysiological activity of the rat heart

Group	Day	ECG					
		RR, мс	HBR, heart beat/min	P(Q)R, мс	(Q)RS, мс	(Q)T,мс	T, мV
Control	0*	165±9	364±7	50,0±2,0	21,5±1,0	109±2	0,14±0,010
	30	165±10	364±6	51,0±2,5	23,0±1,0	108±3	0,12±0,004
PVP,	0	153±8	392±7	52,0±2,5	22,5±1,0	102±1	0,13±0,02
350 mg/kg	30	164±9	366±7	55,0±3,0	22,0±1,0	104±4	0,11±0,02
C_{60}/PVP,	0	171±9	351±7	53,5±1,5	23,5±1,0	104±3	0,14±0,01
350 mg/kg	30	158±6	379±10	51,5±2,5	21,5±1,0	104±2	0,11±0,02
PVP,	0	161±3	373±20	53,5±1,5	21,5±1,0	109±2	0,14±0,02
700 mg/kg	30	160±3	375±20	59,0±3,0	21,0±1,0	111±4	0,11±0,02
C_{60}/PVP,	0	161±7	373±9	52,5±1,0	24,0±1,0	106±2	0,12±0,02
700 mg/kg	30	167±5	359±12	52,5±2,4	23,5±1,0	105±3	0,13±0,02

* - before beginning of injections; values are means ± SEM

C_{60}/PVP and PVP did not change the main parameters of protein, lipid and carbohydrate exchange (glucose, TG, HDL-Chol, TP, A, G).

3.2.3 Electrophysiological activity of heart

Complex C_{60}/PVP and PVP did not influence on the processes of excitation and conduction in heart muscle. Their introduction in the doses of 350 and 700 mg/kg did not affect the duration of P(Q)R, (Q)T, (Q)RS intervals and the T-wave amplitude (Table 3). The substances did not cause the rhythm disturbances, had no effect on the excitement and conduction in cardiac muscle and did not cause the development of myocardial ischemia.

3.2.4 The excretory function of kidneys

In all rats there were no changes in daily diuresis on the 30th day of experiment. No significant differences in specific gravity, pH, protein, sugar, glucose/acetone, and microscopy of urine sediment were observed (Table 4).

Table 4 Effect of C_{60}/PVP and PVP on diuresis and microscopy of urine sediment

Index	Groups				
	Control	PVP, 350 mg/kg	C_{60}/PVP, 350 mg/kg	PVP, 700 mg/kg	C_{60}/PVP, 700 mg/kg
Urine					
Specific gravity, unit	1.008±0.002	1.010±0.002	1.012±0.002	1.015±0.002	1.013±0.002
pH	7.5±0.4	7.3±0.2	7.5±0.2	7.5±0.2	7.0±0.4
Protein, g/l	0.64±0.1	0.67±0.12	0.62±0.03	0.84±0.05	0.77±0.09
Glucose/acetone	-/-	-/-	-/-	-/-	-/-
Diuresis					
1 h	23.8±5.2	10.5±4.5	10.5±5.5	26.3±6.1	18.6±5.2
2 h	70.5±5.8	56.3±9.9	66.4±6.9	71.1±9.7	74.3±4.6
3 h	84.4±6.5	70.3±8.1	91.7±6.1	81.9±9.2	87.9±5.1
4 h	87.5±5.2	73.5±7.8	91.7±6.1	81.9±9.2	91.4±4.9
5 h	92.9±4.4	75.8±7.6	95.7±5.2	87.7±6.2	94.1±4.9
6 h	94.4±5.5	79.5±6.3	97.8±6.1	90.3±7.0	94.4±4.9
24 h	112.3±4.9	108.2±14.9	134.7±8.7	122.5±4.7	124.3±6.7
Microscopic analysis of urine					
Epithelium	0-1-2	0-1-2-3	0-1-2-4	0-1-2-3	0-1-2-3
Leucocytes	0-1-2-3	0-1-2-5	0-1-2	0-1-2-4	0-1-2-4
Erythrocytes	un 0-1-2-3	un 0-1-2	un 0-1-2	0-1-2	un 0-1-2-3
Bacteria	un-1-2-3	un-1-2-3	un-1-2-3	1-2-3	1-2
Tripel phosphate	1-2-3	1	un-1-2-3	1-2-3	1

values are means ± SEM

Injections of C_{60}/PVP or PVP for 30 days did not lead to the changes in the content of urea in the blood serum (Table 2 and 4).

3.2.5 Morphological study of internal organs

PVP itself and C_{60}/PVP complex were not accompanied by fatty degeneration, necrobiosis and necrosis of hepatocytes. The absence of sclerotic changes also excludes the chronic toxic injury. The appearance of mild swelling of the cytoplasm with indications of granular dystrophy was observed only in animals treated with PVP in a dose of 700 mg/kg. Small focal infiltrates of portal lymphoid occurred in all rats, except control.

There were no morphological disturbances of typical architectonic of cortical and medullar layers of the kidneys. In all rats glomerular structure and the convoluted tubules were normal.

Significant hyperplasia of follicles and focal myeloid metaplasia of the pulp were typical structural features of the spleen of rats injected with the test compounds at both doses.

4. Discussion

The possibility of usage of C_{60} in medicine is an important problem of modern medicinal

chemistry and pharmacology. Fullerene C_{60} has very low solubility in water and other biologically acceptable solvents. Therefore it is necessary to use water-soluble complexes or derivatives of C_{60} for medical application and to study their biological effects.

Biological activity and toxicity of such C_{60} complexes, depend on the content of C_{60}, on the molecular weight of polymer which forms the complex, and the tendency of complex to aggregate. C_{60} aggregation tendency is often not taken into consideration, however, numerous data indicate its high influence on the biological effects (Piotrovsky *et al*. 2008, 2011). Naturally, different complexes have different physicochemical properties as well as different biological effects; therefore it is necessary to study their toxicity *in vivo* in each specific case.

C_{60}/PVP complex (C_{60} 5000-6000 ppm, PVP 20 kDa) was synthesized according to Krakovyak *et al.* (2006). The degree of aggregation of the C_{60} molecules in C_{60}/PVP, as was confirmed by UV spectra data (adsorption maximum 333-334 nm), is not high. Membranotropic properties of C_{60}/PVP complex define its antiviral activity (Piotrovsky and Kiselev 2004). C_{60} molecule can also move from the C_{60}/PVP complex to albumin molecule (Schavlovsky *et al*. 2008). This, in turn, may lead to the fact that the distribution of fullerene in the body tissue in the form of fullerene-albumin complex will differ significantly from the distribution and biological effects of all other preparations. In addition, C_{60} release from water-soluble complexes and its penetration into the membrane apparently leads to disappearance of its photodynamic action that gives to the cells resistance to ultraviolet radiation (Kato *et al.* 2014).

These data indicate that the mechanism of biological action of C_{60}/PVP can differ from the biological action of pristine C_{60} (Gharbi *et al*. 2005, Mori *et al*. 2006) or the C_{60} solution in olive oil (Baati *et al*. 2012).

To receive more knowledge about the safety of C_{60}/PVP we examined its acute and sub-acute toxicity after ip administration. LD_{50} of C_{60}/PVP was found to be 7,9±1,0 g/kg. In the study of sub-acute toxicity the doses were selected on the basis of data for acute toxicity. After daily administration of C_{60}/PVP complex for 30 days a wide range of parameters characterizing the structural and functional state of different organs was estimated

Our results demonstrated that the ip administration of the C_{60}/PVP complex did not cause any abnormalities in the state and behavior of the animals. The weight of internal organs of experimental and control rats were similar. The damaging action of the complex on the main parameters of metabolism was not observed. Complex C_{60}/PVP did not affect the processes of excitation and conduction in the heart muscle. The excretory function of the kidneys was normal. The typical architectonic of cortical and medullar layers of the kidneys, glomerular structure and the convoluted tubules remained without alteration. Previously, we showed also that the C_{60}/PVP has no citotoxicity (Kiselev *et al*. 1998).

Yamashita *et al*. (2013) had shown that after 7 days of exposure to oral administration of C_{60}/PVP complex, prepared using PVP 60 kDa with 3000 ppm of C_{60}, this complex did not show any toxic effects, so it can be considered safe for oral application. Unfortunately the data of UV spectra of this complex is not available. However the spectral characteristics of the complexes C_{60}/PVP depend on the PVP molecular mass and fullerene content. Our experience of the work with such complexes shows that complex prepared with PVP 60 kDa has a maximum of adsorption about 340-345 nm, that indicates the high aggregation level of molecules (Krakovjak *et al*. 2005). We used PVP 20 kDa and the aggregation of fullerene molecules was not high. It allowed us to administer complex C_{60}/PVP intraperitoneally to study its toxicity. It is necessary to note, that even at higher concentrations that were used in our experiments, the high bioavailability of C_{60} under ip administration, and long-term observation period no toxic effect was observed.

In the preliminary study of the C_{60}/PVP toxicity it was shown that the PVP itself (1 ml 10% solution, ip) had insignificant, but rather distinct injuring action on various organs (lungs, liver, spleen, kidneys, adrenals, testicles, and thymus). However, C_{60} in C_{60}/PVP complex reduced negative side effects of PVP. It means, that C_{60} has physiological activity, which permits to decrease undesirable effects of the PVP (Popov *et al*. 2008). This is in agreement with literature data (Tsuchiya *et al*. 1996, Podol′ski *et al*. 2002, 2004).

Based on the above results, we can conclude that at ip administration the C_{60}/PVP complex is non-toxic and can be safe for mammals. However among a great number of works, which show the harmlessness of the fullerene itself and the C60/PVP complex, there is only one paper in which it was shown that C_{60}/PVP complex caused abnormalities of embryos after ip injection to pregnant mice and we must take in mind the embryotoxicity which was shown in this work (Tsuchiya *et al*. 1996).

Fullerenes as some other carbon nanostructures can be considered natural substances, and humanity has confronted them over the whole history of its existence (Bang *et al*. 2004, Murr and Soto 2005). In modern conditions, the frequency of contact with rather high concentrations of carbon nanostructures has increased and requires careful study of their safety, considering the form in which these structures are used.

5. Conclusions

The obtained data indicate that C_{60} in the form of the C_{60}/PVP complex has no damaging effect on:

- Mass body of animals and weight of internal organs.
- Hematological and biochemical blood indices.
- Activity of enzyme-markers.
- Main parameters of protein, carbohydrate and lipid metabolism.
- Excretory function of kidneys.
- Liver parenchyma, kidneys and spleen tissue.

Thus water-soluble C_{60}/PVP does not have acute and sub-acute, that suggests further study of its chronic toxicity to establish the possibility of its application in medicine .

Conflict of interests

The authors declared no potential conflict of interests with respect to the research , autorship and/or publication of this paper.

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References

Andrievsky, G.V., Kosevich, M.V., Vovk, O.M., Shelkovsky, S. and Vashchenko, L.A. (1995), "On the production of an aqueous colloidal solution of fullerene", *J. Chem. Soc., Chem. Commun.*, 1281-1282.

Anilkumar, P., Lu, F., Cao, L., Luo, P.G., Liu, J.H., Sahu, S., Tackett. K.N., Wang. Y. and Sun, Y.P. (2011), "Fullerenes for applications in biology and medicine", *Curr. Med. Chem.*, **18**(14), 2045-59.

Anish, G., Jack, B.H., John, B. and Vander, S. (2004), "Size analysis of single fullerene molecules by electron microscopy", *Carbon*, **42,** 1907-1915.

Aoshima, H., Saitoh, Y., Ito, S., Yaman, S. and Miwa, N. (2009), "Safety evaluation of highly purified fullerenes (HPFs): based on screening of eye and skin damage", *J. Toxicol. Sci.*, **4**, 555-562.

Aoshima, H., Yamana, S., Nakamura, S. and Mashino, T. (2010), "Biological safety of water-soluble fullerenes evaluated using tests for genotoxicity, phototoxicity, and pro-oxidant activity", *J. Toxicol. Sci.*, **35**(3), 401-409.

Aschberger, K., Johnston, H.J., Stone, V., Aitken, R.J., Tran, C. L., Hankin, S.M., Peters, S.A.K. and Christensen, F.M. (2010), "Review of fullerene toxicity and exposure - Appraisal of a human health risk assessment, based on open literature", *Regulatory Toxicology and Pharmacology*, **58**, 455-473.

Baati, T., Bourasset, F., Gharbi, N., Njim, L., Abderrabba, M., Kerkeni, A., Szwarc, H. and Moussa, F. (2012), "The prolongation of the lifespan of rats by repeated oral administration of [60]fullerene", *Biomaterials*, **33**, 4936-4946.

Bakry, R., Vallant, R.M., Najam-ul-Haq, M., Rainer, M., Szabo, Z., Huck, C.W. and Bonn, G.K. (2007), "Medicinal applications of fullerenes", *Int. J. Nanomedicine*, **2**(4), 639-649.

Bang, J.J., Guerrero, P.A., Lopez, D.A., Murr, L.E. and Esquivel, E.V. (2004), "Carbon nanotubes and other fullerene nanocrystals in domestic propane and natural gas combustion streams", *J. Nanosci. Nanotechnol.*, **4,** 716-718.

Bensasson, R.V., Bienvenue, E., Dellinger, M., Leach, S. and Seta, P. (1994), "C60 in Model Biological Systems. A Visible-UV Absorption Study of Solvent-Dependent Parameters and Solute Aggregation", *J. Phys. Chem.*, **98,** 3492-3500.

Brant, J., Labille, J, Bottero, J.Y. and Wiesner, M.R. (2006), "Characterizing the impact of preparation method on fullerene cluster structure and chemistry", *Langmuir*, **22**, 3878-3885.

Dugan, L.L., Lovett, E.G., Quick, K.L., Lotharius, J., Lin, T.T. and O'Malley, K.L. (2001), "Fullerene-based antioxidants and neurodegenerative disorders", *Parkinsonism Relat. Disord.*, **7**(3), 243-246.

Dugan, L.L., Turetsky, D.M., Du, C., Lobner, D., Wheeler, M., Almli, C.R., Shen, C.K.F., Luh, T.Y., Choi, D.W. and Lin, T.S. (1997), "Carboxyfullerenes as neuroprotective agents", *PNAS*, **94,** 9434-9439.

Dugan, L.L., Turetsky, D.M., Du, C., Lobner, D., Wheeler, M., Almli, C.R., Shen, C.K.F., Luh, T.Y., Choi, D.W., Lin, T.S., McEwen, C.N., McKay, R.G. and Larsen, B.S. (1992), "C60 as a Radical Scavenger", *J. Am. Chem. Soc.*, **114**, 4412-4414.

Gharbi, N., Pressac, M., Hadchouel, M., Szwarc, H., Wilson, S.R. and Moussa, F. (2005), "Fullerene is an in vivo powerful antioxidant with no acute or sub-acute toxicity", *Nano Letters*, **5**, 2578-2585.

Guldi, D.M., Rahman, G.M.A., Sgobba, V. and Ehli, C. (2006), "Multifunctional molecular carbon materials-from fullerens to carbon nanotubes", *Chem.Soc Rev.*, **35**, 471-487.

Hu, Z., Zhang, C., Huang, Y., Sun, S., Guan, W. and Yao, Y. (2012), "Phgotodynamic anticancer activities of water-soluble C_{60} derivatives and their diological consequences in a Hela cell line", *Chemico-Biological Interactuons*, **195**, 86-94.

Inui, S., Aoshima, H., Nishiyama, A. and Itami, S. (2011), "Improvement of acne vul garis by topical fullerene application: unique impact on skin care", *Nanomedicine: Nanotechnology, Biology, and Medicine*, **7**, 238-241.

Jafvert, C.T., Kulkarni, P.P. (2008), "Buckminsterfullerene's (C60) octanol-water partition coefficient (K ow) and aqueous solubility", *Environ. Sci. Technol.*, **42**(16), 5945-5950.

Johnston, H.J., Hutchison, G.R., Christensen, F.M., Aschberger, K. and Stone, V. (2010), "The biological mechanisms and physicochemical characteristics responsible for driving fullerene toxicity", *J. Toxicol. Sci.*, **114**(2), 162-182.

Kato, S., Aoshima, H., Saitoh, Y. and Miwa, N. (2009), "Biological safety of lipofullerene composed of squalane

and fullerene-C60 upon mutagenesis, photocytotoxicity, and permeability into the human skin tissue", *Basic Clin. Pharmacol. Toxicol*., **104,** 483-487.

Kato, S., Aoshima, H., Saitoh, Y. and Miwa, N. (2010), "Fullerene-C_{60}/liposome complex: Defensive effects against UVA-induced damages in skin structure, nucleus and collagen type I/IV fibrils, and the permeability into human skin tissue", *J. Photochem. Photobiol. B: Biology*, **98,** 99-105.

Kato, S., Aoshima, H., Saitoh, Y. and Miwa, N. (2014), "Fullerene-C60 derivatives 14prevent UV-irradiation/ TiO2-induced cytotoxicity on keratinocytes and 3D-skin tissues through antioxidant actions", *J. Nanosci. Nanotechnol.*, **14**(5), 3285-91.

Kiselev, O., Kozeletskaya, K., Melenevskaya, E., Vinogradova, L., Kever, E., Klenin, S., Zgonnik, V., Dumpis, M. and Piotrovsky L.B. (1998), "Antiviral activity of fullerene C60 with poly(N-vinylpyrrolidone) complex", *Mol. Mat*., **11**, 121-124.

Kolosnjaj, J., Szwarc, H. and Moussa, F. (2007), "Toxicity studies of fullerenes and derivatives", *Adv. Exp. Med. Biol.*, **620**,168-180.

Krakovjak, M.G., Anufrieva, E.V., Anan'eva, T.D. and Nekrasova, T.N. (2006), "Water-soluble complexes of poly(N-vinylamides) of various structures with C60 and C70 fullerenes", *Polym. Sci. Ser. A*, **48**, (6), 590-595.

Krakovjak, M.G., Anufrieva, E.V., Anan'eva, T.D., Piotrovskij, L.B. and Nekrasova, T.N. "Water-soluble complex of fullerene with poly-n-vinylpyrrolidone and method for preparing these complexes", 10.07.2005 Patent RU 2255942.

Krustic, P.J., Wassermann, E., Keizer, P.N., Morton, J.R. and Preston, K.F. (1991), "Radical reaction of C60", *Science*, **254**, 1183-1185.

Lens, M. (2009), "Use of fullerenes in cosmetics", *Recent patents on Biotechnol*., **3**, 118-123.

Liu, Q., Cui, Q., Li, X.J. and Jin, L. (2014), "The applications of buckminsterfullerene C_{60} and derivatives in orthopedic research", *Connect. Tissue Res*., **55**(2), 71-9.

Long, Y.Ch., Lub, F.J. and Linc, J.T. (1995), "Free radical scavenging activity of water-soluble fullerenols", *J. Chem. Soc., Chem. Commun.*, 1283.

Mori, T., Takada, H., Ito, S., Matsubayashi, K., Miwa, N. and Sawaguchi T. (2006), "Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis", *Toxicology*, **225**, 48-54.

Moussa, F., Trivin, F., Ceolin, R., Hadchouel, V., Sizaret, P.Y., Greugny, V., Rassat, A., Szwarc, H. (1996), "Early effects of C_{60} administration in Swiss mice: a preliminary account for *in vivo* C_{60} toxicity", *Ful. Sci. Technology*, **4**(1), 21-29.

Murayama, H., Tomonoh, S., Alford, J.M. and Karpuk, M.E. (2005), "Fullerene Production in Tons and More: From Science to Industry", *Fuller Nanotub Car N*, **12**, (1), 1-9.

Murr, L.E. and Soto, K.F. (2005), "A TEM study of soot, carbon nanotubes, and related fullerene nanopolyhedra in common fuel-gas combustion sources", *Mater.Characteriz*., **55**, 50-65.

Nielsen, G.D., Roursgaard, M., Jensen, K.A., Poulsen, S.S. and Larsen, S.T. (2008), "In vivo biology and toxicology of fullerenes and their derivatives", *Basic. Clin. Pharmacol. Toxicol*., **103**, 197-208.

Park, E., Roh, J., Kim, Y. and Park, K. (2010), "Induction of inflammatory responses by carbon fullerene (C60) in cultured RAW264.7 cells and intraperitoneally injected mice", *Toxicol. Res*., **26**(4), 267-273.

Piotrovsky, L.B. (2006), "Biological activity of pristine fullerene C60", Carbon Nanotechnology, Ed. L. Dai. Elsevier, Dayton. USA.

Piotrovsky, L.B., Dumpis, M.A., Litasova, E.V., Eropkin, M.Y., Eropkina, E.M. and Kiselev, O.I. (2011), "Dependence of biological effects of fullerene C60 *in vitro* from the type of preparations", *Fuller Nanotub Car N*, **19**(1), 147-153.

Piotrovsky, L.B. and Kiselev, O.I. (2004), "Fullerenes and viruses", *Fuller Nanotub Car N*, **12**(1), 397-403.

Piotrovsky, L.B., Dumpis, M.A., Poznyakova, L.N. and Kiselev, O.I. (2005), "Influence of complexes fullerene C_{60} with γ-CD on the morphology influenza viruses", *Questions of Biological, Medicinal and Pharmaceutical Chemistry*, **3**, 21-24.

Piotrovsky, L.B., Eropkin, M.Y., Eropkina, E.M., Dumpis, M.A. and Kiselev, O.I. (2008), "Biological effects in cell cultures of fullerene C60: dependence on aggregation state", *Medicinal Chemistry and Pharmacological Potential of Fullerenes and Carbon Nanotubes*, Cataldo F., Da Ros T., Springer.

Piotrovsky, L.B. (2013), *Essays on Nanomedicine*, Europe House, Saint Petersburg, Russia.

Podolski, I.Y., Kondratjeva, E.V., Shcheglov, I.V., Dumpis, M.A. and Piotrovsky, L.B. (2002), "Fullerene C60 complexed with poly (N-vinylpyrrolidone) prevents the disturbance of long-term memory consolidation", *Phys. Solid States*, **44**, 552-554.

Podolski, I.Y., Kondratjeva, E.V., Gurin, S.S., Dumpis, M.A. and Piotrovsky, L.B. (2004), "Fullerene C60 complexed with poly(N-vinylpyrrolidone) prevents the disturbance of long-term memory consolidation induced by cycloheximide", *Fuller Nanotub Car N*, **12**, 421-424.

Popov, V.A., Tyunin, M.A., Zaitseva, O.B., Karaev, R.H., Sirotinkin, N.V., Dumpis, M.A. and Piotrovsky, L.B. (2008), "C60/PVP complex - no toxicity after introperitoneal injection to rats", *Fuller Nanotub Car N*, **16**(5), 693-697.

Prozovskii, V.B. and Prozorovskaia. M.P. (1980), "Table method of determining the ED50 (LD50) of substances with low biological activity". *Farmacol. Toksikol*., **43**(6), 733-735.

Schavlovsky M.M., Soloviev K.S., Dumpis M.A., Litasova E.V. and Piotrovsky L.B. (2008), "Complexes of fullerene C60 with blood plasma proteins", Nanotechnology in Biology and Medicine, Ed. E.V. Schlyachto, SPb, Russia.

Sushko, M.L., Klenin, S.I., Piotrovsky, L.B. and Poznyakova, L.N. (1999), "Light scattering in water solution of fullerene -containing polymers. Part 2. Effect of the molecular weight of the scatter molecules", *Tech. Phys. Lett.*, **25**, 778-779.

Takada, H., Mimura, H., Xiao, L., Islam, R.M., Matsubayashi, K., Ito, S. and Miwa, N. (2006), "Innovative antioxidant: Fullerene (INCI #: 7587) is as radical sponge on the skin. Its high level of safety, stability and potential as premier anti-aging and whitening cosmetic ingredient", *Fuller Nanotub Car N*, **14**(2-3), 335-341.

Takahashi, M., Kato, N., |Doi, Y., Hagiwara, A., Hiirata-Koizumi, M., Ono, A., Kubota, R., Nishimura, T. and Hirose, A. (2012), "Sub-acute toxicity study with fullerene C60 in rats", *J. Toxicol. Sci.*, **37**(2), 353-361.

Tsuchiya, T., Oguri, I., Yamakoshi, Y.N. and Miyata, N. (1996), "Effect of [60]fullerene on the chondrogenesis in mouse embryonic limb bulb cell culture system", *Full. Sci. Technol*., **4**, 989-999.

Tsuchiya, T., Oguri, I., Yamakoshi, Y.N. and Miyata, N. (1996) "Novel harmful effects of fullerene on mouse embryos in vivtro and in vivo", *FEBS Lett.*, **393**, 139-145.

Xiao, Y. and Wiesner, M.R. (2012), "Characterization of surface hydrophobicity of engineered nanoparticles", *J. Hazard. Mater*., **215-216**, 146-151.

Yamakoshi, Y.N., Yagami, T., Fukuhara, K., Fukuhara, K., Sueyoshi, S. and Miyata, N. (1994), "Solubilization of fullerenes into water with polyvinylpyrrolidone applicable to biological tests", *J. Chem. Soc., Chem. Commun*., 517-518.

Yamashita, K., Yoshioka, Y., Pan. H., Taira, M., Ogura, T., Nagano, T., Aoyama, M., Nagano, K., Abe, Y., Kamada, H., Tsunoda, S.I., Aoshima, H., Nabeshi, H., Yoshikawa, T. and Tsutsumi, Y. (2013), "Biochemical and hematologic effects of polyvinylpyrrolidone-wrapped fullerene C_{60} after oral administration", *Pharmazie*, **68**(1), 54-7.

Vengerovich, N.G., Tuynin, M.A., Antonenkova, E.V., Konshakov, Y.O., Bolehan, A.V., Zaytceva, O.B., Stukov, A.V., Boyarkin, M.N. and Popov, V.A. (2012), "Biological activity of fullerene C_{60} nanobiocomposites", *Immunology*, **12**, 161-177.

Zhao, B., He, Y.Y., Chignell, C.F., Yin, J.J., Andley, U. and Roberts, J.E. (2009), "Difference in phototoxicity of cyclodextrin complexed fullerene [(γ-CyD)2/C_{60}] and its aggregated derivatives toward human lens epithelial cells", *Chem. Res. Toxicol*., **22**(4), 660-66.