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DYNAMIC EFFECTS OVER PLASMA REDOX BALLANCE FOLLOWING SUBCUTANEOUS INJECTION OF SINGLE WALLED CARBON NANOTUBES FUNCTIONALIZED WITH SINGLE STRAND DNA

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Single-walled carbon nanotubes (SWCNT) represent a material holding significant potential for biomedical applications. However, toxicity concerns have been issued. Still, evidences regarding their toxic effects following subcutaneous administration are still scarce. We aimed to evaluate the dynamics of plasma redox equilibrium within the first 48 hours after subcutaneous injection of SWCNT functionalized with single strand DNA (ss-DNA-SWCNT).

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Experimental

390 mg L^{-1} concentrated solution was obtained through water sonication. Experiments were performed on Wistar rats. The test group (n=10) was administered 1.5 ml of ss-DNA-SWCNT solution through subcutaneous (sc.) injection. At seriate intervals from administration (3, 6, 24, and 48 hours, respectively), collection of blood was performed. Control group (n=10) was similarly injected 1.5 ml NaCl 0.9% and blood was collected at 3 hours from exposure. Malondialdehyde (MDA), protein carbonyls (PC), hydrogen donor ability (HD), sulfhydryl groups (SH) and nitric oxide (NO) levels were assessed.

Results. Significant increase of MDA, PC, NO in plasma was obtained following administration, peaking at 6 hours from administration ($p<0.05$). As a result of exposure, both HD and SH levels decreased, reaching bottom levels at 24 hours from injection ($p<0.05$). A strong positive correlation was detected between dynamics of NO and MDA plasma levels ($p<0.01$). Also, a strong negative correlation was obtained between plasma levels of PC and HD ($p<0.01$).

Conclusions. Subcutaneous administration of SWCNT functionalized with single strand DNA induces activation of plasma oxidative stress mechanisms.

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1. Introduction

Single-walled carbon nanotubes(SWCNT) represent a material holding significant potential for biomedical applications. However, recent reports have launched concerns regarding the safety of the material. Consensus among authors exists regarding the dependency of toxic effects on nanomaterial physical and chemical properties : purity, cristallinity, functional groups [1]. *In vitro* reports have demonstrated the ability of SWCNT to stimulate the cytokine production [2]. The increase of reactive oxygen species (ROS) generation and activation of nuclear transcription factor-kB was also reported in cell culture experiments [3]. Going a step further, *in vivo* experiments have demonstrated the pro-oxidative properties of SWCNT after dermal exposure [4] and intra-peritoneal injection [5]. In case of SWCNT pharyngeal aspiration [6] and after lung inhalation, induction of inflammation, fibrosis, oxidative stress and mutagenesis have also been reported [7]. However, consistent results concerning subcutaneous injection of SWCNT are still scarce. We aimed to evaluate the dynamics of plasma redox equilibrium within the first 48 hours after subcutaneous injection of SWCNT.

2. Experimental

2.1. Synthesis of SWCNT was performed using an inductive heating method (acetylene, 850^{o}C, Fe:Mo:MgO catalyst -1.4:0.14:98.46 wt %) as previousely described [8, 9]. Following synthesis, nanotubes were further purified using a Soxhlet device (refluxing with HCl (1:1), 24 hrs), water washed (up to neutral PH) and dried (120 ^{o}C, 12 hrs). Purity of obtained SWCNT was>98%, as shown by thermo galvanometric (TGA) analysis.

2.2. SWCNT functionalization. In short, ss-DNA- SWCNT was obtained from 40 mg SWCNT and 40 ml ss-DNA 0.1% solution (Sigma-Aldrich Chemicals GmbH - Germany) through sonication (Sonics, Vibra-Cell VC 505, 500 Watt, 20 kHz) for 15 minutes (ice-water bath, 30% amplitude). The mixture was consequently centrifuged (Sigma-Aldrich, 1 hr, 4000g), with removal of undispersed SWCNT. For both the collected supernatant and ss-DNA 0.1% solution, optical absorption measurements were performed (Jasco V-570 UV-vis-NIR Spectrophotometer, 1/40 dilution, 1 cm quarts cuvette). The estimative concentration of ss-DNA-SWCNT solution was 390 mg L^{-1}.

2.3. Experimental model. Two groups of Wistar rats (190±10g) entered the experiment. The test group (n=10) was administered 1.5 ml of ss-DNA-SWCNT solution through subcutaneous (s.c.) injection. At seriate intervals from administration (3, 6, 24, and 48 hours, respectively), collection of blood was performed. Control group (n=10) was similarly injected 1.5 ml NaCl 0.9% and blood was collected at 3 hours from exposure.

The experimental protocol was approved by the Local Ethics Committee, as part of a National Research Grant [NANOCITOX: 42-112/2008].

2.4.Oxidative stress parameters assays

2.4.1. Malondialdehyde (MDA) assay was performed using an already described method [10]. In short, 1 hour heating of plasma samples (boiling water bath, 10mM 2-thiobarbituric acid in 75mM K_2HPO_4, pH3) followed by cooling and N-butanol extraction were completed. Spectrofluorimetric measurement was performed (Perkin Elmer LS45B Spectrofluorimeter, 520 nm excitation**,** 534 nm emission)

2.4.2. Protein carbonyls (PC) assay was done using an already published technique [11]. Briefly, plasma samples were treated with 2,4-dinitrophenyl-hydrazine(10nM, in 2.5N HCl, 1 hour, in the dark, 21 ^{o}C). Next, addition of 20% trichloroacetic acid (on ice) was performed followed by 3 repetitive washing with 1:1(v/v) ethyl acetate-etanol mixture and protein pellet dissolvation (6M guanidine hydrochloride). Determination of carbonyl content (355 nm

spectrophotometer reading, absorption coefficient ε for aliphatic hydrazones = 22,000$M^{-1}cm^{-1}$; formula: $C=Abs_{355}$ x 45.45.) as well as protein concentration (280 nm, standard curve) were performed.

2.4.3.Hidrogen Donors Ability Assay

Using an already published method [12] plasma samples were diluted (10 mM phosphate buffer, pH 7.4), and further added 0,1 mM 1,1-diphenyl-picrylhydrazyl (DPPH) solution (methanol, 30 min, room temperature). Reading was performed using a spectrophotometer (517 nm, control: DPPH in 10 mM phosphate buffer, pH 7.4), followed by final estimation of concentration (formula: Reducing activity (%)= [(absorbance of control sample-absorbance of test sample)/absorbance of the control]*100).

2.4.4.Sulfhydryl groups assay

As it has been previously described [13] 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB or Ellman reagent) was used for marker level measurements. Dilution of plasma samples with Tris (0.25M)-EDTA buffer (20mM), pH 8.2 was done, followed by absorbance reading (412nm, DTNB 10mM solution in methanol, prior to and 15 min post reaction) and final concentration estimation (absorptivity of 13,600$M^{-1}cm^{-1}$).

2.4.5.Nitrites / nitrates assays - Griess rection

For NO measurement, the two-step Griess reaction was used.[14] Briefly, in the first step the reduction of nitrate to nitrite was performed (glucose-6-phosphate (2.5mM)+ glucose-6-phosphate dehydrogenase (400U/l); NADPH-dependent nitrate reductase (200U/l) in 14mM sodium phosphate buffer, pH7.4; NADPH solution (0.02mmol)). Successively, Griess reagent treatment was performed (incubation; $ZnSO_4$ (30%) protein precipitation; reaction with equal parts of 0.1g N-1-naphthyl ethylene diamine hydrochloride in 100 ml water and 1g sulfanyl-amide in 100 ml 5% orthophosphoric acid). Absorbance reading (540 nm) and final concentration estimation was done (by comparison to nitrite-standard curve of Na NO_2 solutions).

2.5.Statistical Methods:

Normality of continuous data was assessed by means of Kolmogorov-Smirnov Test. Variance across time intervals was tested using Kruskall-Wallis test. Consequently, post-hoc comparisons were performed by means of Mann-Whitney U Test. For reduction of alpha error risk, Bonferroni adjustment was performed with significance threshold establishment at $p<0.01$ level. Correlation between dynamic evolution of various parameters was assessed using Spearman's r correlation coefficient. SPSS 17.0 (Chicago, Il, USA) statistical package was used for all data analysis.

3. Results

There was no death or detectable adverse effects among animals in study, following ss-DNA-SWCNT administration.

3.1. Oxidative markers

MDA plasma level recorded a significant increase, peaking at 6 hours post administration ($p<0.01$, Table 1). Median level for 6 hours post exposure represented 191.6% of median control group. After this time point marker levels presented a decreasing trend, reaching values of 150% of controls at 24 hours (Fig. 1). At the end of the 48 hours of follow-up, MDA values were as low as 50% of control levels.

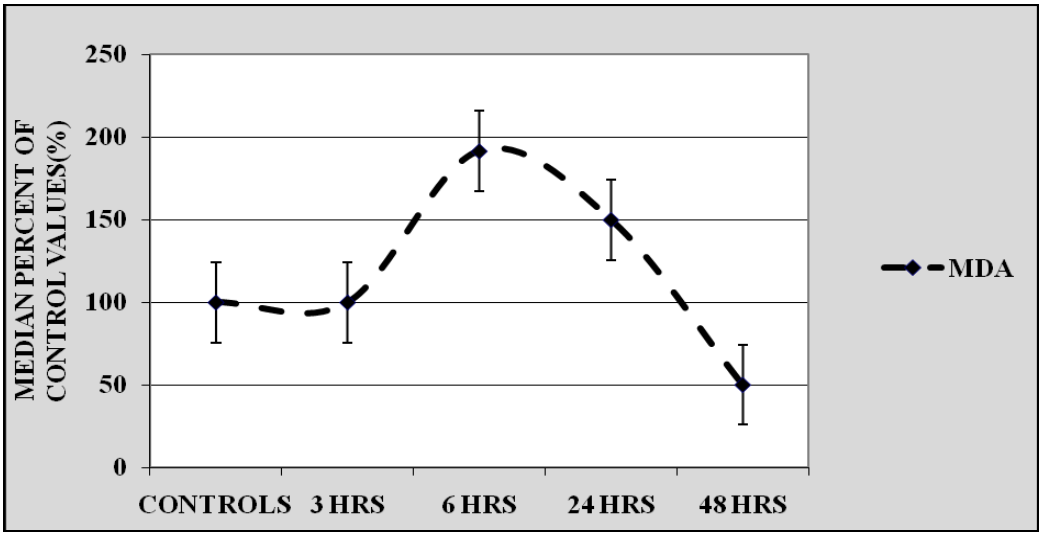

Fig. 1. Dynamics of plasma MDA level after subcutaneous administration of ss-DNA-SWCNT. Points represent median percent values calculated using control values as baseline (100%). Error bars represent standard error.

PC plasma levels also attained the highest levels at 6 hours from administration (191.6% of controls). At this time point, levels were significantly elevated as compared to controls ($p<0.01$, Table 1). Although PC values recorded a slow decreasing trend, levels recorded at 24 and 48 hours remained significantly higher as compared to controls (183.3% and 175.0% of controls, respectively, Figure 2).

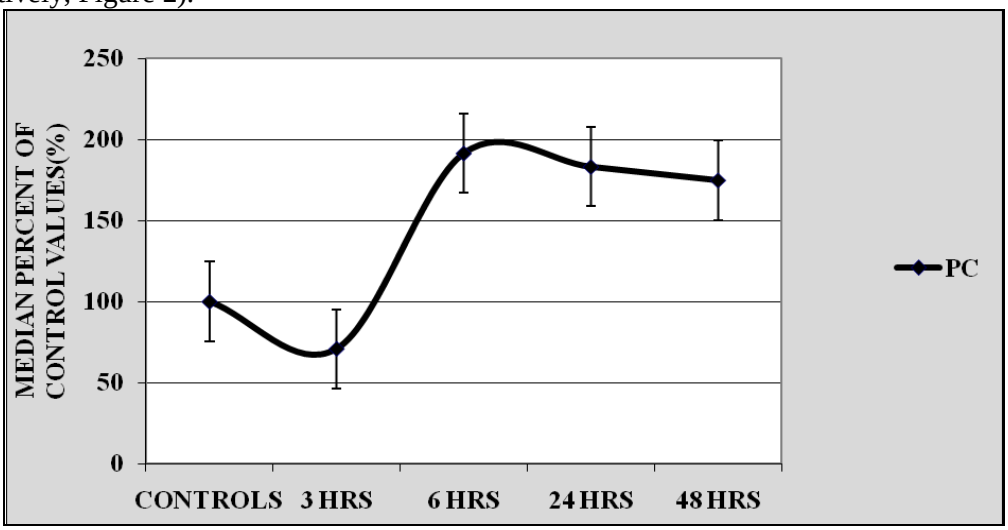

Fig. 2. Dynamics of plasma PC level after subcutaneous administration of ss-DNA-SWCNT. Points represent median percent values calculated using control values as baseline (100%). Error bars represent standard error.

Similarly, the peak for NO plasma level was obtained at 6 hours post- exposure. Median recorded value of the marker was as high as 281.5% of controls ($p<0.01$, Table 1). After the 6 hour interval from exposure, values presented a decreasing tendency reaching 90.5% of control values at the end of the follow-up interval (Fig. 3).

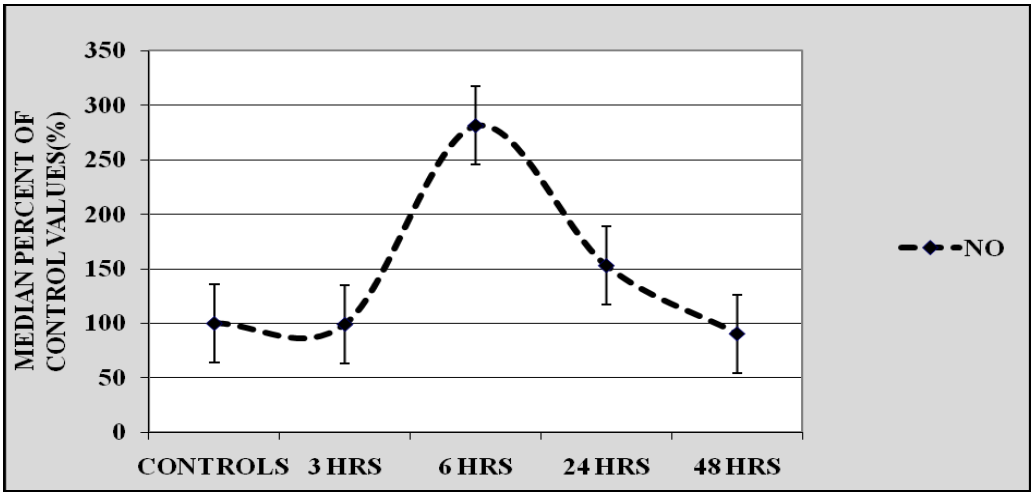

Fig. 3. Dynamics of plasma NO level after subcutaneous administration of ss-DNA-SWCNT. Points represent median percent values calculated using control values as baseline (100%). Error bars represent standard error.

3.2. Antioxidant markers

HD plasma levels decreased starting from 6 hours from exposure (79.88% of control levels) and reached the minimum level at 24 hours (79.2% of controls) (Table 1). At the end of the follow-up interval levels recorded a slight increase to reach a median level of 88.24% of median control level (Fig. 4).

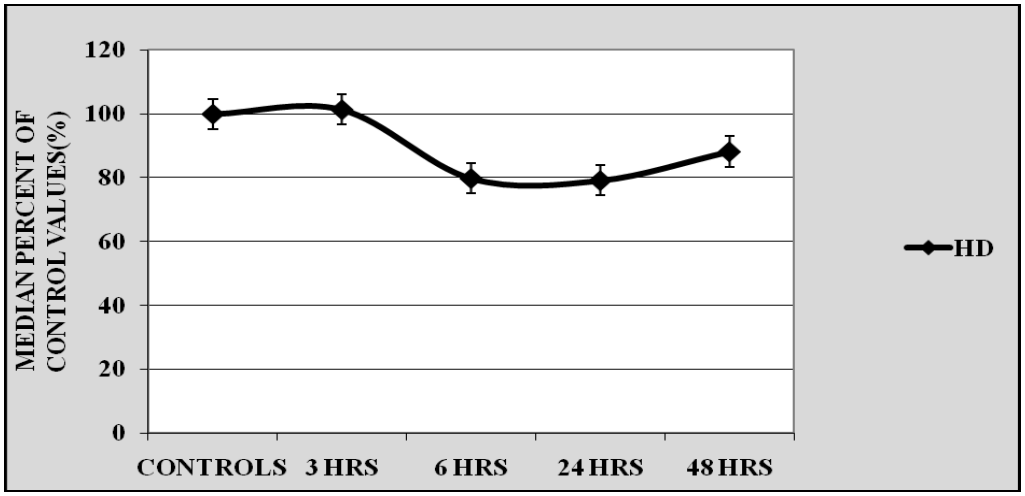

Fig. 4. Dynamics of plasma HD level after subcutaneous administration of ss-DNA-SWCNT. Points represent median percent values calculated using control values as baseline (100%). Error bars represent standard error.

Plasma levels of SH groups recorded a homogenous decreasing tendency, from 80.6% at 3 hours from administration, to 55.7% at 6 hours and down to 39.8% of control values at 24 hours from exposure (Table 1). After the last 24 of the follow-up period, levels remained as low as 42.3% of controls.

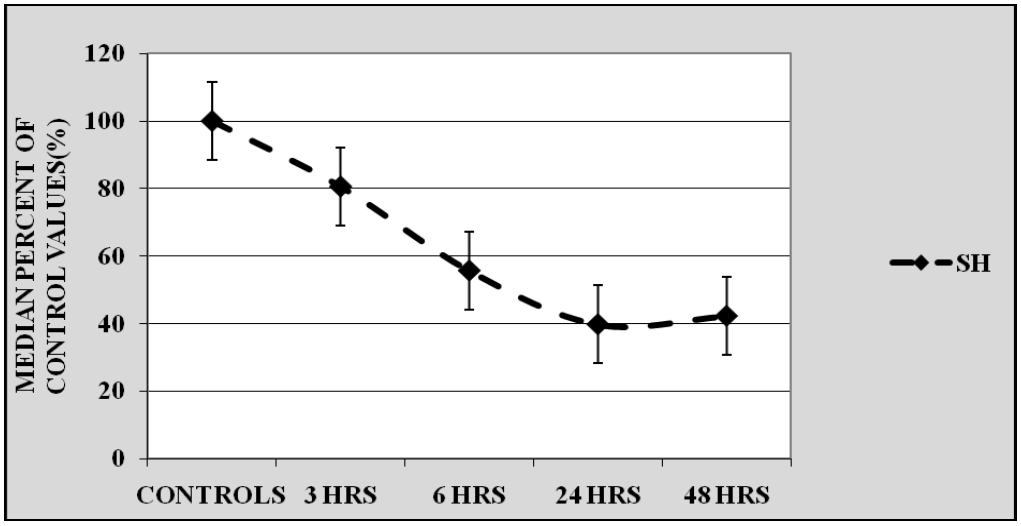

Fig. 5. Dynamics of plasma SH level after subcutaneous administration of ss-DNA-SWCNT. Points represent median percent values calculated using control values as baseline (100%). Error bars represent standard error.

Table 1. Absolute values of plasma markers at seriate time points.

Marker	**Control group**	**Test group**			
	3 hours	**3 hours**	**6 hours**	**24 hours**	**48 hours**
MDA (nmol ml^{-1})	1.2(0.2)	1.2(0.5)	2.3(0.1)*	1.8(0.5)*	0.6(0.3)*
PC (nmol mg protein^{-1})	1.2(0.5)	0.8(0.2)	2.3(0.7)*	2.2(0.6)*	2.1(0.3)*
NO (µmol l^{-1})	4.8(4.8)	4.7(5.1)	13.7(3.8)*	7.4(2.6)*	4.4(5.5)
HD (inhibition%)	44.2(6.2)	44.8(6.7)	35.3(5.2)*	35.0(5.7)*	39.0(5.8)*
SH (micromol ml^{-1})	0.2(0.1)	0.16(0.2)	0.11(0.4)*	0.08(0.3)*	0.08(0.2)*

Values represent median (range) values.
* *represent significant differences as compared to control values ($p<0.01$).*

Dynamic inter-correlations.

A strong positive correlation was detected between dynamics of NO and MDA plasma levels, respectively ($r=0.975$, $p=<0.01$). Also, dynamic evolution of PC demonstrated a significant negative correlation with dynamic evolution of HD ($r=-0.900$, $p<0.01$).

3. Discussions

Within this study we evaluated the dynamic effects following subcutaneous administration of ss-DNA-SWCNT over plasma oxidant/antioxidant balance. For characterization of oxidative injury, the first analyzed plasma marker was malondialdehyde. The marker represents one of the secondary oxidation products similar with isoprostanes, 4-4-hydroxy-2-,3-trans-nonenal(HNE) or oxysterols [15]. Along with MDA, levels of protein carbonyl have also been determined for quantifying the cellular ROS-induced damage involving amino-acid residues oxidation [16]. Both plasma markers revealed a bimodal dynamic pattern within the first 48 hours from ss-DNA-SWCNT administration, with a first phase of increasing levels in the first 6 hours, followed by a second phase with a decreasing trend onwards.

The parallel evolution of nitric oxide with that of MDA, sustained by the significant positive correlation between the two markers suggest the involvement of NO in oxidative attack. It has previousely been stated that NO is able to react with superoxide resulting in peroxynitrite formation, a very reactive free radical incriminated for high rates of lipoperoxidation [17, 18, 19]. Our results clearly link the dynamics of NO with that of peroxidation products like MDA,

demonstrating NO role in oxidative damage following subcutaneous injection of ss-DNA-SWCNT.

By contrast, the antioxidant defense markers recorded bottom levels at 24 hours. The first marker, -SH groups, could have gone through a consumption process within the 0-24 hours interval from administration. It has been stated that thiol groups can play several protective roles such as: free radical scavenging, especially important for $HO^{\cdot}$[20], restoration of molecules to the original state after oxidative injury [21] or constituting a cofactor for key antioxidant enzymes [20]. The same consumption process could explain the dynamics of hydrogen donors. The marker gathers the whole antioxydant capacity of protector systems (thiol groups, vitamines, etc) [22]. Its obtained dynamic pattern for the first 48 hours reveals the decrease in the global antioxydant defense as a response to the ss-DNA-SWCNT administration.The signifficant inverse correlation between HD and PC dynamics suggests the predominance of oxidative attack orientation towards proteic structures.

Present results rise medium and long-time safety concerns. Studied markers suggest a transitory oxidative state and tend to return to baseline status at the end of the 48 hours from administration. However, at 48 hours from administration, some of the analysed markers still remain significantly different as compared to baseline levels. PC levels still remained as high as 175% of control levels, while SH levels in test group continued to be below control levels (42.3% of baseline). Those findings results suggest that effects may go beyond the proposed follow-up interval. Also, another concern is that primary oxidation products could react with MDA, resulting in further oxidative damage [22]. Due to self-activation, a vicious pathogenetic circle can appear, thus resulting in outburst of oxidative stress-based pathologies. Counteracting measures might be needed for long time safe subcutaneous administration of ss-DNA-SWCNT.

4. Conclusions

Subcutaneous administration of SWCNT functionalized with single strand DNA induces activation of plasma oxidative stress mechanisms. Long term effects need to be further analyzed and might call for counteracting measures.

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