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In vitro Mammalian Chromosomal Aberration Test of Fullerene-C60

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Fullerene-C60의 포유류 배양세포를 이용한 염색체이상시험

김수진, 임경택※, 조해원, 한정희, 김현영, 양정선

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요 약

Fullerene의 유전독성을 평가하기 위하여 Chinese hamster유래의 난소유아세포(CHO-K1 cell)를 이용하여 직접법(－S9)과 대사활성화법(＋S9 mix)의 염색체이상시험을 실시하였다. 시험물질은 1% CMC 나트륨염의 현탁액(1% CMC 용액)에 희석하여 조제하였다.

대사활성화를 시키지 않은 직접법의 염색체이상시험에서 24시간 투여군은 8단계의 농도(0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10 mM)로 투여하여 실시하였다. 투여 농도 증가에 따른 염색체이상의 빈도가 증가하는 양상이 나타나지 않았다. 48시간의 투여군에서는 8단계의 농도(0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10 mM)로 투여하여 실시하였는데 투여 농도 증가에 따른 염색체이상의 빈도가 증가하는 양상이 나타나지 않았다. 배수체의 염색체이상은 직접법에서 관찰되지 않았다.

대사활성화법을 이용하여 6시간 시험물질을 투여한 시험에 있어서는 8단계의 용량단계(0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10 mM)를 설정하였는데 투여 농도가 증가함에 따른 염색체이상빈도의 증가양상이 관찰되지 않았다.

이상의 결과를 종합할 때 본 시험물질은 본 시험 조건하에서 CHO-K1세포에서 대사활성화를 시켰을 때 염색체이상을 유발하지 않는 것으로 판단된다.

Key words : Fullerene, chromosomal aberration, *in vitro*, Chinese Hamster Ovary

INTRODUCTION

It being increased the necessity of hazard assessment with nano-materials because of the increase of exposure frequency to workers as developing the nano-industries.

Nanotoxicology, the toxicology of nanoparticles (particles ＜100 nm diameter) that appear to have some toxic effects that are unusual and not seen with larger particles. Nanoparticles can be divided into

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combustion-derived nanoparticles (like diesel soot), manufactured nanoparticles like carbon nanotubes and naturally occurring nanoparticles from volcanic eruptions, atmospheric chemistry etc.

Nanoparticles seem to have some different properties from larger particles that are known to have pathogenic effects, like asbestos or quartz. These differences may be a result of their size. They have a larger surface area per unit mass and this means that in some cases they may have more pro-inflammatory effects in, e.g. the lungs. Moreover some may be able to translocate from their site of deposition to distant sites such as the blood and the brain.

It was performed the *in vitro* mammalian chromosomal aberration test of Fullerene (CAS No. 99685-96-8) which was not sufficient its definite information but increased the necessity of hazard assessment. Moreover it would make use of this toxicological information of Fullerene for carbon nano-material treated workers' right of known, to prepare or update the MSDS of it.

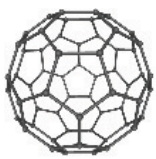
Fullerene is called in total carbon molecules connected with pentagonal or hexagonal like a soccer ball, was discovered first in left soot in which the laser shoot to graphite. It means mainly the carbon cluster C60 linked as a ball. It forms a nano-ball with 1 nm diameter, be originated in an architect, B. Fuller (1895～1983) designed a dome looks like this nano-material. It also called "buckyball" which originated in his name. The researches came to be active since it produced with the techniques of Donald Huffman and Wolfgang Krätschmer in 1990 (Kroto *et al.*, 1985)

It is applying to eliminate the static electricity by adding to resin with its hydrophobicity. It is researched to produce stronger and sharper cutting devices or plastics from fullerene. The physicochemical properties of fullerene that the molecular weight is 720.64, density is 1.6 g/cm^3, flash point is over 94°C (Appendix 1). The general symptom of exposure is irritation to eyes or respiratory system.

It was detected Fullerene in blood, spleen, liver after i.p injection (Moussa *et al.*, 1997), also in embryo and yolk sac across the placental barrier (Tsuchiya *et al.*, 1996). It decreases the glutathione activity as the effect to liver metabolic enzymes in human and rat *in vitro* (Iwata *et al.*, 1998). It was observed that no effect to DNA synthesis as a level of initiation and promotion of carcinogenesis in subacute skin toxicity. It did not increase the epithelial tumor but promoted the enzyme activity for this growth (Nelson, 1993). Sera *et al.* (1996) observed the mutagenicity for treatment the Fullerene with light to *Salmonella* in metabolic activated condition (+S9), Zakharenko (1993) did not observe any mutagenicity in *E. coli* and larva of Drosophila *in vitro*. Kamat *et al.* (1998) reported the Fullerene (12.5 μg C60-cyclodextin) induces the oxidative damages to liver microsomes *in vitro*. These damages could control with antioxidants or free radical scavengers etc. Oberdörster (2005) researched the effect of Fullerene to peroxide production in fishes, observed the significantly increase of lipid peroxidation from brain of fishes by 0.5 ppm Fullerene.

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used

Appendix 1. General information of Fullerene.

Chemical names	Fullerene		
Chemical names	Buckminsterfullerene (5,6)Fullerene-C60-Ih Footballene		
Maker	Sigma-Aldrich Co.		
Molecular formula	C_{60} 		
Molecular weight	720.64		
Purity	99.9 %	Lot No.	08005JE
CAS No.	99685-96-8	Characteristic on room temperature	Black powder

*Fullerene is a nano-scaled chemical which connect with hexagonal plane of carbon atoms like a soccer ball.

system is a co-factor supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254, or a combination of phenobarbitone and β-naphthoflavone. The post-mitochondrial fraction is usually used at concentrations in the range from 1～10% v/v in the final test medium. The condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilize more than one concentration of post-mitochondrial fraction. A number of developments, including the construction of genetically engineered cell lines expressing specific activating enzymes, may provide the potential for endogenous activation. The choice of the cell lines used should be scientifically justified (e.g., by the relevance of the cytochrome P450 isoenzyme for the metabolism of the test substance) (OECD, 1997).

Despite its increasing use, the available genotoxicity data on Fullerene are still controversial. Thus it was evaluated to determine the genotoxicity or mutagenicity with *in vitro* mammalian chromosomal aberration test. Typical nanoparticles that have been studied are titanium dioxide, alumina, zinc oxide, carbon black, and carbon nanotubes, and "nano-C_{60}". But it had not performed the *in vitro* mammalian chromosomal aberration test with Fullerene.

MATERIALS AND METHODS

1. Cells and chemicals

The cell which used in test is the cultivated CHO-K1 (Chinese hamster ovary fibroblast) origins. The cell was obtained from the Korean Cell Line Bank (KCLB 10061). It was cultured in F-12 medium (GIBCO BRL, USA, Lot No. 1244774) with 5% CO_2 at 37°C, subcultured every 2～4 days.

It was used 1% CMC sol'n (Sigma, USA, Lot No. 046K0050) as a solvent according to the result of solubility test. Positive control was used mitomycin C (MMC) (Sigma, USA, Lot No. 094K0493) and cyclophosphamide (CPA) (Sigma, USA, Lot No. 076K1050). Negative control was used 1% CMC solution. For the metabolic activated system, the S9 (MOLTOX™, Annapolis, Maryland, USA, Lot No. 2151) was used within 6 months after manufactured.

Fullerene, a nano-material was obtained (Appendix 1) and performed the image analysis for confirm the nano-scale with Transmission Electron Microscope (TEM, H-7100FA, x50～x600,000, 25～125 KV, Hitachi, Japan)

2. *In vitro* mammalian chromosomal aberration test

This study was performed according to OECD guidelines for the testing of chemicals (OECD, 1997) (*In vitro* Mammalian Chromosomal Aberration Test. Ref. OECD TG473) and Ishidate's report (Ishidate *et al.*, 1985).

For cell proliferation suppression test, the 8 dosages (0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5.0 and 10.0 mM respectively) were used. For direct method (24 and 48 hour treatment), it was cultured for about 3 days from the aliquot with 2×10^4～4×10^4 cells in 60 mm diameter plate. For metabolic activated method (6 hour treatment), it was cultured with same as direct method. The slides of chromosomal sample were made from 5 mL media aliquot with 18 hour supplementary culture after removal of media and washing the cell layer with 5 mL fresh media.

The main test was performed with dosages established by cell proliferation suppression/preliminary test. After 24 and 48 hour of fullerene treat to each plates, treat the 0.2 μg/mL Colcemid® (GIBCO BRL, USA, Lot No. 1402494), separate the metaphase cell after 2 hours and take away with centrifuge at 1,000 rpm for 5 min.

It was made the chromosome samples after 3 times fixing with the Carnoy's sol'n (acetic acid : ethanol = 1 : 3) and counted the abnormalities after dyeing with 5% Giemsa (Merck, NJ, USA, Lot No. OB513429) sol'n for 5 min. Two samples were made in each plate.

It was observed 100 cell in metaphase per plate and

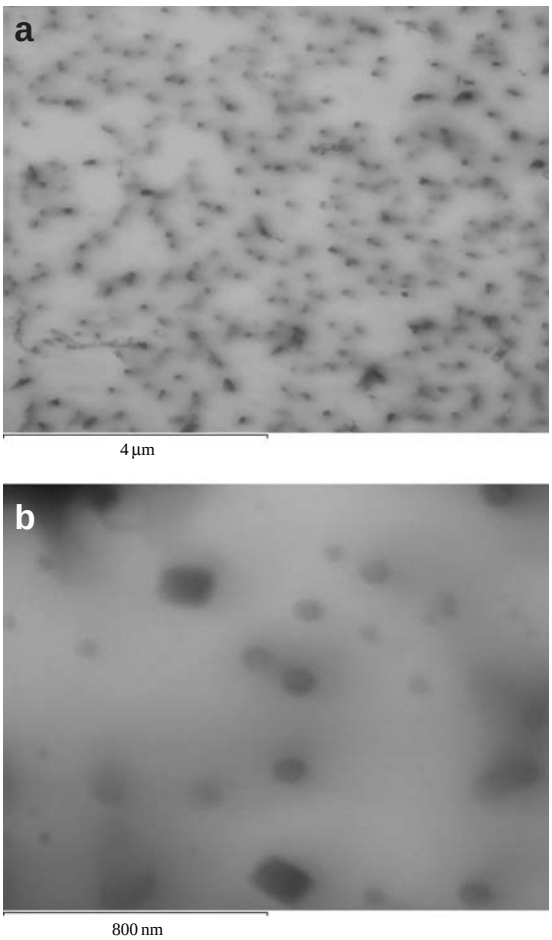


Fig. 1. The Transmission Electron Microscope images of Fullerene dissolved with CMC. (a) The TEM image of Fullerene dissolved with CMC, ×10,000 (Scale bar 4 μm). (b) The TEM image of Fullerene dissolved with CMC, ×50,000 (Scale bar 800 nm).

classified as the structural abnormalities (gap of chromatid or chromosome; *g*, cutting of chromatid; *ctb*, exchange of chromatid; *cte*, cutting of chromosome; *csb*, exchange of chromosome; *cse* and the others) and numerical abnormalities (*pol*). However the retest was performed in case that it was not confirmed the dosage dependency and the ratio of chromosomal aberration was unusually high in control solvent. It was not performed the statistical analyses of the results.

Table 1. Cell proliferation suppression test with Fullerene

	Concentration (mM)	Cell proliferation ratio (%)	
		24 hr treatment	48 hr treatment
Direct method (−S9)	0 (solvent)	100	100
	0.078	114.07	77.61
	0.156	93.24	112.06
	0.313	60.12	84.03
	0.625	74.71	99.02
	1.25	95.68	89.82
	2.5	108.25	61.84
	5.0	93.83	69.58
	10.0	82.40	54.01

	Concentration (mM)	Cell proliferation ratio (%)
		6 hr treatment
Metabolic activated method (+S9)	0 (solvent)	100
	0.078	112.54
	0.156	60.85
	0.313	83.85
	0.625	83.51
	1.25	100.70
	2.5	105.12
	5.0	72.34
	10.0	105.23

RESULTS

Before the main study, we performed the image analysis of the Fullerene for confirm their nano-scale with Transmission Electron Microscope, and we confirmed it as a nano-scale (Fig. 1).

1. Test of suppression with cell proliferation

The ratios of cell proliferation for the dosage of test were shown in Table 1 that 60.12%, 74.71% at 0.313 mM and 0.625 mM for 24 hour treatment with direct method respectively. Moreover it shows that 77.61%, 61.84%, 69.58% and 54.01% at 0.078 mM, 2.5 mM, 5.0 mM and 10.0 mM for 48 hour treatment. In direct method, the 8 dosages (0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5.0 and 10.0 mM respectively) were used, and it is based on the notice from NIER (National Institute of Environmental Research, Korea; http://www.nier.go.kr) of Korea that the maximum concen-

Table 2. Chromosomal aberration test (direct method, 24 hr treatment)

Treatment	Time of treatment (hr)	Conc. of treatment (mM)	Obs. cell No.	No. of diploid		No. and ratio of chromosomal structure abnormality								Slide No.	Decision
						Gap	Chromatid		Chromosome		Etc.	Total			
				Decision		g	ctb	cte	csb	cse		−g	+g		
Control solvent (1% CMC)	24	0	101	0		0	0	0	0	0	0	0	0	1	
			101	0	−	2	0	0	0	0	0	0	2	12	−
			202	0		2 (1)	0	0	0	0	0	0	2 (1)		
Test material	24	0.078	103	0		1	0	0	0	2	0	2	3	5	
			100	0	−	1	1	0	0	0	0	1	2	14	−
			203	0		2 (1)	1 (0.5)	0	0	2 (1)	0	3 (1.5)	5 (2.5)		
		0.156	102	0		1	0	0	0	0	0	0	1	2	
			100	0	−	0	0	0	0	4	0	4	4	9	−
			202	0		1 (0.5)	0	0	0	4 (2)	0	4 (2)	5 (2.5)		
		0.313	100	0		2	0	0	0	0	0	0	2	3	
			100	0	−	1	0	0	0	0	0	0	1	11	−
			200	0		3 (1.5)	0	0	0	0	0	0	3 (1.5)		
		0.625	100	0		3	0	0	0	0	0	0	3	13	
			104	0	−	4	0	2	0	0	0	2	6	17	−
			204	0		7 (3.5)	0	2 (1)	0	0	0	2 (1)	9 (4.4)		
		1.25	100	0		2	0	1	0	0	0	1	3	4	
			101	0	−	3	0	0	0	1	0	1	4	6	−
			201	0		5 (2.5)	0	1 (0.5)	0	1 (0.5)	0	2 (1)	7 (3.5)		
		2.5	101	0		4	0	1	0	0	0	1	5	10	
			100	0	−	1	0	0	0	0	0	0	1	19	−
			201	0		5 (2.5)	0	1 (0.5)	0	0	0	1 (0.5)	6 (3)		
		5	100	0		3	0	0	0	1	0	1	4	7	
			101	0	−	2	0	0	0	0	0	0	2	16	−
			201	0		5 (2.5)	0	0	0	1 (0.5)	0	1 (0.5)	6 (3)		
		10	100	0		1	0	1	0	0	0	1	2	18	
			100	0	−	0	0	0	0	2	0	2	2	20	−
			200	0		1 (0.5)	0	1 (0.5)	0	2 (1)	0	3 (1.5)	4 (2)		
Posit. contr. (MMC)	24	0.0004 (mg/mL)	100	0		15	14	29	1	7	0	51	66	8	
			100	0	−	21	14	39	0	1	0	54	75	15	+
			200	0		36 (18)	28 (14)	68 (34)	1(0.5)	8 (4)	0	105 (52.5)	141 (70.5)		

g, gap; ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange; ( ), average

Table 3. Chromosomal aberration test (direct method, 48 hr treatment)

Treatment	Time of treatment (hr)	Conc. of treatment (mM)	Obs. cell No.	No. of diploid		No. and ratio of chromosomal structure abnormality								Slide No.	Decision
						Gap	Chromatid		Chromosome		Etc.	Total			
					Decision	g	ctb	cte	csb	cse		−g	+g		
Control solvent (1% CMC)	48	0	101	0		0	0	0	0	0	0	0	0	2	
			100	0	−	0	0	0	0	0	0	0	0	7	−
			201	0		0	0	0	0	0	0	0	0		
Test material	48	0.078	105	0		0	0	0	0	0	0	0	0	15	
			100	1	−	0	0	1	0	0	0	1	1	17	−
			205	1 (0.5)		0	0	1	0	0	0	1 (0.5)	1 (0.5)		
		0.156	100	0		0	0	0	0	0	0	0	0	9	
			100	0	−	0	1	0	0	0	0	1	1	6	−
			200	0		0	1	0	0	0	0	1 (0.5)	1 (0.5)		
		0.313	106	1		1	0	0	0	0	0	0	1	1	
			100	0	−	1	0	0	0	0	0	0	1	11	−
			206	1 (0.5)		2	0	0	0	0	0	0 (0)	2 (1.0)		
		0.625	103	1		1	0	1	0	0	0	1	2	3	
			100	0	−	3	0	1	0	0	0	1	4	18	−
			203	1 (0.5)		4	0	2	0	0	0	2 (1.0)	6 (3.0)		
		1.25	107	0		0	0	0	0	0	0	0	0	12	
			100	0	−	1	0	0	0	0	0	0	1	19	−
			207	0		1	0	0	0	0	0	0	1 (0.5)		
		2.5	100	1		0	0	0	0	0	0	0	0	4	
			102	0	−	1	0	0	0	0	0	0	1	16	−
			202	1 (0.5)		1	0	0	0	0	0	0	1 (0.5)		
		5	103	0		1	0	0	0	0	0	0	1	5	
			100	0	−	0	0	0	0	0	0	0	0	10	−
			203	0		1	0	0	0	0	0	0	1 (0.5)		
		10	100	0		0	0	0	0	0	0	0	0	14	
			101	1	−	0	1	0	0	0	0	1	1	20	−
			201	1 (0.5)		0	1	0	0	0	0	1 (0.5)	1 (0.5)		
Positive contr. (MMC)	48	0.0004 (mg/mL)	103	0		20	24	37	0	0	0	61	81	8	
			100	0	−	18	21	34	0	0	0	55	73	13	+
			203	0		38	45	71	0	0	0	116 (57.1)	154 (75.9)		

g, gap; ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange; ( ), average

Table 4. Chromosomal aberration test (metabolic activated method, 6 hr treatment)

Treatment	Time of treatment (hr)	Conc. of treatment (mM)	Obs. cell No.	No. of diploid		No. and ratio of chromosomal structure abnormality								Slide No.	Decision
						Gap	Chromatid		Chromosome		Etc	Total			
					Decision	g	ctb	cte	csb	cse		−g	+g		
Control solvent (1% CMC)	24 (6+18)	0	100	0		1	1	2	0	0	0	3	4	7	
			100	0	−	0	0	4	0	1	0	5	5	15	−
			200	0		1 (0.5)	1 (0.5)	6 (3)	0	1 (0.5)	0	8 (4)	9 (4.5)		
Test material	24 (6+18)	0.078	125	0		1	0	2	0	0	0	2	3	1	
			100	0	−	0	1	5	0	0	0	6	6	17	−
			225	0		1 (0.5)	1 (0.5)	7 (3.5)	0	0	0	8 (3.6)	9 (4.0)		
		0.156	100	0		0	0	1	0	0	0	1	1	3	
			100	0	−	2	0	3	0	3	0	6	8	5	−
			200	0		2 (1)	0	4 (2)	0	3 (1.5)	0	7 (3.5)	9 (4.5)		
		0.313	120	0		0	0	1	0	0	0	1	1	2	
			100	0	−	2	2	4	0	0	0	6	8	12	−
			220	0		2 (1)	2 (1)	5 (2.5)	0	0	0	7 (3.2)	9 (4.1)		
		0.625	111	0		0	0	2	0	0	0	2	2	14	
			103	0	−	1	0	2	0	0	0	2	3	16	−
			214	0		1 (0.5)	0	4 (2)	0	0	0	4 (1.9)	5 (2.3)		
		1.25	101	0		0	1	3	0	0	0	4	4	4	
			101	0	−	1	2	2	0	0	0	4	5	8	−
			202	0		1 (0.5)	3 (1.5)	5 (2.5)	0	0	0	8 (4)	9 (4.5)		
		2.5	103	0		2	0	2	0	0	0	2	4	10	
			102	0	−	0	0	3	0	0	0	3	3	18	−
			205	0		2 (1)	0	5 (2.5)	0	0	0	5 (2.4)	7 (3.4)		
		5	132	0		0	1	4	0	0	0	5	5	11	
			101	0	−	0	0	4	0	0	0	4	4	20	−
			233	0		0	1 (0.5)	8 (4)	0	0	0	9 (3.9)	9 (3.9)		
		10	100	0		0	0	2	0	0	0	2	2	13	
			102	0	−	1	0	2	0	0	0	2	3	19	−
			202	0		1 (0.5)	0	4 (2)	0	0	0	4 (2)	5 (2.5)		
Positive contr. (CPA)	24 (6+18)	0.01 (mg/mL)	100	0	−	10	10	43	0	0	0	53	63	6	
			100	0		9	9	30	0	0	0	39	48	9	+
			200	0		19 (9.5)	19 (9.5)	73 (36.5)	0	0	0	92 (46)	111 (55.5)		

g, gap; ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange; ( ), average number

tration is over 5 mg/mL in case of the cellular toxicity was not recognized. Cell proliferation shows that 60.85%, 72.34% at 0.156 mM and 5.0 mM for 6 hour treatment with metabolic activated method.

2. Chromosomal aberration test

The ratios of chromosomal aberration in direct method were shown in Table 2 and Table 3. It was not observed any diploid at any concentrations for 24 hour treatment. The structural chromosomal aberrations were below 5.0% in both −gap and +gap. It had no dependency between chromosomal aberrations and dosages. Each one diploid was observed in 0.078 mM, 0.313 mM, 0.625 mM, 2.5 mM, 10 mM for 48 hour treatment.

The ratios of chromosomal aberration in metabolic activated method were shown in Table 4. All results were the same as in direct method. It is known that the Fullerene do not induced any chromosomal aberrations in both direct method (24 hour and 48 hour treatment) and metabolic activated method for 6 hour treatment with using CHO-K1 cell. From these results, it was evaluated by the facts that it is "positive" with the ratio of chromosomal aberration is over 10%, and it had dependency between chromosomal aberrations and dosages.

DISCUSSION

From this study, it was resulted that Fullerene did not induce the chromosomal aberration both in direct method (24 hours and 48 hours treatment) and metabolism activated method for 6 hour treatment in test with CHO-K1 cell.

In vitro testing provides a cost-effective means for the studies; cell culture experiments are well suited for developing mechanistic models to inform material development. It is expected this work will set a standard for future efforts to characterize the environmental and health impacts of other classes of engineered nanoparticles.

Ultimately, such proactive toxicological studies will be vital to ensure the nanomaterials design process yields both effective and safe technologies.

The smaller a particle, the greater its surface area to volume ratio and the higher its chemical reactivity and biological activity. The greater chemical reactivity of nanomaterials results in increased production of reactive oxygen species (ROS), including free radicals (Nel, 2006). ROS production has been found in a diverse range of nanomaterials including carbon fullerenes, carbon nanotubes and nanoparticle metal oxides. ROS and free radical production is one of the primary mechanisms of nanoparticle toxicity; it may result in oxidative stress, inflammation, and consequent damage to proteins, membranes and DNA (Nel, 2006).

It was reported that the alkaline (pH > 13) SCGE assay is very effective to detect base oxidation, DNA single strand breakage (SSB) with ROS. It is applied that the study with cellular repair activity with DNA segments for reveal or amplify the genotoxic effects of nanoparticles, and that measuring the repair activity with DNA repair enzymes or inhibition of DNA damage by antioxidants (Jekinson *et al.*, 1999), and quantitative measurements of specific oxidative base (Collins *et al.*, 1993; Covallo *et al.*, 2003).

Moreover how these nanoparticles behave inside the organism is one of the big issues that need to be resolved. The behavior of nanoparticles is a function of their size, shape and surface reactivity with the surrounding tissue. They could cause overload on phagocytes, cells that ingest and destroy foreign matter, thereby triggering stress reactions that lead to inflammation and weaken the body's defense against other pathogens. Nanoparticles accumulate in organs, another concern is their potential interaction with biological processes inside the body. For instance, this may affect the regulatory mechanisms of enzymes and other proteins.

Nanomaterials are able to cross biological membranes and access cells, tissues and organs that larger-sized particles normally cannot (Holsapple *et al.*, 2005). Nanomaterials can gain access to the blood stream following inhalation (Oberdörster *et al.*, 2005) or ingestion (Hoet *et al.*, 2004). At least some nano-

materials can penetrate the skin (Ryman-Rasmussen, 2006), even larger microparticles may penetrate skin when it is flexed (Tinkle, 2003).

Nanomaterials have proved toxic to human tissue and cell cultures, resulting in increased oxidative stress, inflammatory cytokine production and cell death (Oberdörster *et al.*, 2005). Unlike larger particles, nanomaterials may be taken up by cell mitochondria (Li, 2003) and the cell nucleus (Geiser, 2005; Porter, 2007). Studies demonstrate the potential for nanomaterials to cause DNA mutation (Geiser, 2005) and induce major structural damage to mitochondria, even resulting in cell death (Li, 2003; Savic, 2003). Size is therefore a key factor in determining the potential toxicity of a particle. However it is not the only important factor.

Other properties of nanomaterials that influence toxicity include: chemical composition, shape, surface structure, surface charge, aggregation and solubility (Covallo *et al.*, 2003), and the presence or absence of functional groups of other chemicals (Magrez, 2006). The large number of variables influencing toxicity means that it is difficult to generalize about health risks associated with exposure to nanomaterials, each new nanomaterial must be assessed individually and all material properties must be taken into account.

From this study, we reviewed for genotoxicity testing of nanomaterials that must Know what nanomaterial has been tested and in what form, recognize that nanomaterials are not all the same, and consider uptake and distribution of the nanomaterial, take nanomaterials specific properties into account and use standardized methods and *in vivo* studies to correlate *in vitro* results. And much more learning about the mechanism of nanomaterials genotoxic effects will be necessary.

Experiences with other, non-nano, substances (molecules and larger particles) taught us, that mechanisms of genotoxic effects can be diverse and their elucidation can be demanding, while there often is an immediate need to assess the genotoxic hazard. Thus a practical and pragmatic approach is the use of a battery of standard genotoxicity testing methods covering a wide range of mechanisms. Application of these standard methods to nanomaterials demands, however, several adaptations and the interpretation of results from the genotoxicity tests may need additional considerations (Robert, 2009). This study should help to improve testing of nanomaterials by generally used genotoxicity testing methods as well as investigations on the underlying mechanism and the interpretation of genotoxicity data on nanomaterials.

From all of the results, it was suggested the further investigations to perform such as FLARE assay (Comet assay with repair enzyme as Fpg, Endo III), real time RT-PCR etc. Moreover it would be more useful as a biomarker for chemical risk assessment to perform these tests with many other nanomaterials.

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