書籍

著者氏名	論文タイトル名	書籍名	出版社名	ページ	出版年
Kazuo Isama, Toshie Tsuchiya	Gamma-ray Irradiated Poly(L-lactide) for Bone Repair	Biomaterials in Asia	World Scientific Publishing Company	254-265	2008 出版地 シンガポール
澤田留美、土屋利江	細胞組織医療機器の品質・安全性：ヒト間葉系幹細胞の遺伝子発現を指標とした特性解析	幹細胞の分化誘導と応用	エヌ・ティー・エス	496-508	2009 出版地 東京
土屋利江	フラーレンの毒性	ナノ材料のリスク評価と安全性対	フロンテイア出版	in press	
土屋利江	医療用ナノ材料の国際標準化動向	ナノ材料のリスク評価と安全性対	フロンテイア出版	in press	

雑誌

発表者名	論文タイトル	発表誌名	巻号	ページ	出版年
土屋利江	医療機器の製造販売承認について、特集「コラーゲン・テクノロジーの	Materials Integration	23	67-70	2010
Matsuoka, A., Onfelt, A., Matsuda, Y., Nakaoka, R., Haishima, Y., Yudasaka, M. Iijima, S., Tsuchiya, T.	Development of an in vitro screening method for safety evaluation of nanomaterials	Bio-Med. Mater. Engineering	19	19-27	2009
山田貴史、山越葉子、石川烈、 石川格、土屋利江、松岡厚子	V79細胞および正常ヒトアストロサイトに対するフラーレンとカーボンナノチューブおよびその誘導体の細胞	国立医薬品食品衛生研究所報告	127	39—43	2009
Yamada, T., Nakaoka, R., Sawada, R., Matsuoka, A., Tsuchiya, T.	Effects of intracerebral microinjection of hydroxylated-[60]fullerene on brain monoamine concentrations and locomotor	J. Nanosci. Nanotechnol.	10	604-611	2010
M. Tamai, K. Isama, R. Nakaoka, T. Tsuchiya	Synthesis of novel β-tricalcium phosphate/ hydroxyapatite biphasic calcium phosphate containing niobium ions and evaluation of its osteogenic properties	Journal of Artificial Organs	10	22-28	2007
伊佐間和郎、鹿庭正昭、土屋利江	アクセサリー類を除く金属製品に含有する鉛及びカドミウムの分析調査	中毒研究	21	393-395	2008
中島晴信、沢辺善之、伊佐間和郎、土屋利江	高分子材料中のオクチル酸スズ(2-エチルヘキサン酸スズ)の分析	大阪府立公衆衛生研究所研究報告	46	97-102	2008
伊佐間和郎、鹿庭正昭、土屋利江	キャピラリー電気泳動法によるアルカリ性洗浄剤中のナトリウムイオン、カリウムイオン及びモノエタノールアミンの分析	国立医薬品食品衛生研究所報告	126	71-75	2008
大嶋智子、尾崎麻子、中島晴信、伊佐間和郎、土屋利江	ポリ乳酸プラスチック中の有機スズ化合物の分析	大阪市立環境科学研究所報告	71	21-26	2009
R. Nakaoka, Y. Yamakoshi, K. Isama, T. Tsuchiya	Effects of surface chemistry prepared by self-assembled monolayers on osteoblast behavior	Journal of Biomedical Materials Research: Part A	in press		
Ahmed S., Tsuchiya T., Nagahata-Ishiguro M., Sawada R., Banu N., Nagira T.	Enhancing action by sulfated hyaluronan on connexin-26, -32, and -43 gene expressions during the culture of normal human astrocytes.	J Biomed Mater Res A	90(3)	713-719	z
Machida K., Suemizu H., Kawai K., Ishikawa T., Sawada R., Ohnishi Y.,	Higher susceptibility of NOG mice to xenotransplanted tumors.	J. Toxicol. Sci	34(1)	123-127	2009

澤田留美、松岡厚子、松田良枝、土屋利江	ヒト間葉系幹細胞の in vitro 培養期間中の変化について―c-myc をターゲットとした遺伝子発現解析と染色体異常解析―	薬学雑誌	128(12)	1851-1856	2008
Tam P. S. Y., Sawada R., Cui Y., Matsumoto A., Fujiwara Y.	The metabolism and distribution of docosapentaenoic acid (n-6) in the liver and testis of growing rats.	Biosci. Biotechnol. Biochem.	72(10)	2548-2554	2008
Yamada T., Sawada R., Tsuchiya T.	The effect of sulfated hyaluronan on the morphological transformation and activity of cultured human	Biomaterials	29	3503-3513	2008
Yamada T., Jung DY, Sawada R., Matsuoka A., Nakaoka R., Tsuchiya T.	Effects intracerebral microinjection and intraperitonealninjection of [60]fullerene on braion functions differ in rats.	J. Nanosci. Nanotechnol.	8(8)	1-9	2008
Yamada T., Jung DY, Sawada R., Tsuchiya T.	Intracerebral microinjection of stannous 2-ethylhexanoate affects dopamine turnover in cerebral cortex and locomotor activity in	J Biomed Mater Res B : Appl Biomater.	87B(2)	381-386	2008
Ito T., Sawada R., Fujiwara Y., Tsuchiya T.	FGF-2 increases osteogenic and chondrogenic differentiation potentials of human mesenchymal stem cells by inactivation of TGF-b	Cytotechnology	56	1-7	2008
Ito T., Sawada R., Fujiwara Y., Seyama Y., Tsuchiya T.	FGF-2 suppresses cellular senescence of human mesenchymal stem cells by down-regulation of TGF-beta2.	Biochem Biophys Res Commun.	359(1)	108-114	2007
澤田留美、伊藤友実、土屋利江	細胞組織医療機器に用いられる幹細胞の品質及び安全性評価につい	薬学雑誌	127(5)	851-856	2007
迫田秀行, 鄭徳泳, 佐藤道夫, 土屋利江, 脇谷滋之, 天正恵治	人工関節の不具合要因分析	日本臨床バイオメカニクス学会誌	29	361	2008
迫田秀行, 鄭徳泳, 佐藤道夫, 土屋利江, 脇谷滋之, 天正恵治	微小試験片を用いた人工関節用UHMWPEの疲労特性評価	日本臨床バイオメカニクス学会誌	29	367	2008
迫田秀行, 石川格, 鄭徳泳, 佐藤道夫, 土屋利江, 脇谷滋之, 天正恵治	微小試験片を用いた高密度架橋ポリエチレンの疲労特性評価	臨床バイオメカニクス	30	263	2009
迫田秀行, 鄭徳泳, 佐藤道夫, 土屋利江, 脇谷滋之, 天正恵治	人工関節の不具合要因分析 第二報 人工股関節	臨床バイオメカニクス	30	319	2009
Tsunoda, M., Yoshida, T., Tsuji, M, Zhang, Y., Sugaya, Y., Inoue, Y., Miki, T, Satoh, T. and Aizawa, Y.	The effects of dibutyltin (DBT) dichloride on the viability and the productions of tumor necrosis factor・and interleukin-12 in murine macrophage cell line, J774.1.	Biomedical Research on Trace Elements	19(1)	67-71	2008
Masayohi Tsuji, Yoko Inoue, Chiemi Sugaya, Masashi Tsunoda, Tsukiko Sugaya, Masami Takahahi, Toshiyasu Yuba, Toshie Tsuchiya, Yoshiharu Aizawa	Higher toxicity of dibutyltin and poly-L-lactide with a large amount of tin but lower toxicity of poly-L-lactide of synthetid artificial dura mater exhibited on murine astrocyte cell line	YAKUGAKU ZASSHI	130	in press	2010
Masashi Tsunoda, Tamae Yoshida, Masayoshi Tsuji, Ying Zhang, Chiiemi Sugaya, Yoko Inoue, Takeo Miki, Toshihiko Satoh, Yoshiharu Aizawa	The effects of dibutyltin (DBT) dichloride on the viability and the productions of tumor necrosis factor a and interleukin-12 in murine macrophage cell line, J774.1.	Biomedical Research on Trace Elements	19	67-71	2008
Mitsugu Todo, Yuji Takahashi Ryuji Nagamine	Stress analysis of artificial knee joints under flexion and rotation	Tribology Online	3	211-215	2008
東藤 貢,長嶺隆二,高橋祐一	PS型人工膝関節における繰り返し屈曲動作ト脛骨インサートの塑性変形の関係について	日本臨床バイオメカニクス学会誌	29	381-387	2008
東藤 貢,長嶺隆二,高橋祐一	医療画像に基づく3次元膝関節モデルの構築と人工膝関節の応力解析への応用	臨床バイオメカニクス	30	339-346	2009

Authors	Title	Journal	Vol	Pages	Year
Makoto Ohta, Naoko Fujimura, Luca Augsburger, Krisztina Barath, Hasan Yilmaz, German Abdo, Karl-Olof Lovblad, Daniel A.	Subtracted Vortex Centers Path Line Method with Cinematic Angiography for Measurement of Flow Speed in Cerebral Aneurysms	Neurological Research	30	251-255	2008
Makoto Ohta、Chuan He、Toshio Nakayama、Akira Takahashi、Daniel A. Rufenacht	Three-Dimensional Reconstruction of a Cerebral Stentusing Micro-CT for Computational Simulation	Journal of Intelligent Material Systems and	19	313 -318	2008
Kazuto Takashima, Shinzo Ota、Makoto Ohta、Kiyoshi Yoshinaka、Toshiharu Mukai	Development of a catheter and guidewire simulator for interventional therapy	日本バイオレオロジー学会誌	22	12-18	2008
K. Srinivas、T. Nakayama、M. Ohta、S. Obayashi、T. Yamaguchi	Studies on Design Optimization of Coronary Stents"Journal of Medical Devices	Journal of Medical Devices	2	011004(1-7)	2008
Lei Liu、Toshiyuki Hayase、Makoto Ohta、Kosuke Inoue	Experimental Validation of Color Doppler Velocity Measurement for Ultrasonic- Measurement-Integrated Simulation of Blood Flow	Journal of Biomechanical Science and Engineering	3	161-175	2008
Toshio Nakayama、Hitoshi Hayase、Koji Tokunaga、Makoto Ohta	Paramater Study of Hemodynamics Simulation at Internal Carotid Stenosis	Journal of Fluid Science and Technology	3	544-552	2008
太田 信	脳動脈瘤用ステントの血流減少効果を解析するために	日本機械学会誌	111	538	2008
Takahashi Kandori、Toshiyuki Hayase、Kousuke Inoue、Kenishi Funamoto、Takanori Takeno、Makoto Ohta、Motoshiro Takeda、Atsushi Shirai	Frictional Characteristics of Erythrocytes on Coated Glass Plates Subject to Inclined Centrifugal Forces	J. Biomech. Eng.	130	051007-1 - 8	2008
L.Augsburger, P.Reymond, E.Fonck, Z.Kulcsar, M. Farhat, M. Ohta, N. Stergiopulos, D.A.Rufenacht	Methodologies to assess blood flow in cerebral aneurysms: Current state of research and perspectives	Journal of Neuroradiology	36	270-277	2009
Makoto Suto, Hiroyuki Kosukegawa, Kaoru Maruta, Makoto Ohta, Kazuyuki Tohji, Balachandran Jeyadevan	Heat diffusion characteristics of magnetic nanoparticles dispersed hydro-gel in alternating magnetic field	Journal of Magnetism and Magnetic Materials	321	3483-3487	2009
Yuang-Seng Tsuei, Yasushi Matsumoto, Makoto Ohta, Toshio Nakayama, Masayuki Ezura, Akira Takahashi	Vertebrobasilar junction fenestration with dumbbell-shaped aneurysms formation: computational fluid dynamics analysis	Surgical Neurology	72	S11-S19	2009
Lei Liu, Hiroyuki Kosukegawa, Makoto Ohta, Toshiyuki Hayase	An isotropic in vitro Vessel Model Using (Vinyl Alcohol) Hydro Gel and Mesh Material	Journal of Applied Polymer Science	in press		
ChangHo Yu, Hiroyuki Kosukegawa, Keisuke Mamada, Kanju Kuroki, Kazuto Takashima, Kiyoshi Yoshinaka, makoto Ohta	Study on Catheter Movement with Poly (vinyl alcohol) Hydrogel for the Development of an In Vitro Tracking System	Journal of Biomechanical Science and Engineering	in press		
Masato Tamai, Ryusuke Nakaoka, Kazuo Isama, Toshie Tsuchiya	Synthesis of novel beta-tricalcium phosphate/hydroxyapatite biphasic calcium phosphate containing niobium ions and evaluation of osteogenic properties	J. Artif. Organs	10	22-28	2007
Shigeyuki Wakitani, Masashi Nawata, Amu Kawaguchi, Takahiro Okabe, Kunio Takaoka, Toshie Tsuchiya, Ryusuke Nakaoka, Hiroyuki Masuda, Kyosuke Miyazaki	Serum keratan sulfate is a promising marker of early articular cartilage breakdown	Rheumatology	46	1652-1656	2007

中岡竜介、松本富美子、宗林さおり、柳橋哲夫、土屋利江	視力補正を目的としないおしゃれ用カラーコンタクトレンズの細胞毒性	国立医薬品食品衛生研究所報告	125	61-64	2007
Takashi Yamada, Duk-Young Jung, Rumi Sawada, Atsuko Matsuoka, Ryusuke Nakaoka, Toshie Tsuchiya	Effects intracerebral microinjection and intraperitoneal injection of [60] fullerene on brain function differ in rats	J. Nanoscience Nanotechno	8	1-9	2008
Bayar Hexig, Ryusuke Nakaoka, Toshie Tsuchiya	Safety evaluation of surgical materials by cytotoxicity test	J. Artif. Organs	11	204-211	2008
迫田秀行、中岡竜介、松岡厚子、土屋利江	三次元スキャフォールドを用いた細胞培養系の評価方法の検討	国立医薬品食品衛生研究所報告	126	76-81	2008
Atsuko Matsuoka, Agneta Önfelt, Yoshie Matsuda, Ryusuke Nakaoka, Yuji Haishima, Masako Yudasaka, Sumio Iijima, Toshie	Development of an in vitro screening method for safety evaluation of nanomaterials	J. Biomed. Mater. Eng.	19	19-27	2009
Takashi Yamada, Ryusuke Nakaoka, Rumi Sawada, Atsuko Matsuoka, Toshie Tsuchiya	Effects of intracerebral microinjection of hydroxylated-[60] fullerene on brain monoamine concentrations and locomotor	J. Nanoscience Nanotechno	10	604-611	2010
Ryusuke Nakaoka, Yoko Yamakoshi, Kazuo Isama, Toshie Tsuchiya	Effects of surface chemistry prepared by self-assembled monolayers on osteoblast behavior	J. Biomed. Mater. Res. A	in press		2010
石川格, 澤田 留美, 加藤幸夫, 辻 紘一郎, 邵 金昌, 山田 貴史, 加藤 玲子, 土	新無血清培地STK2のヒト間葉系幹細胞増殖における有用性について	YAKUGAKU ZASSHI	129(3)	381-384	2009
石川格、迫田秀行、菅野信彦、松岡厚子、土屋利江	光学式3Dデジタイザによる抜去人工股関節UHMWPEライナーの摩耗測定	日本臨床バイオメカニクス学会誌	submitted		

III　研究成果の刊行物・別刷

Effects Intracerebral Microinjection and Intraperitoneal Injection of [60]Fullerene on Brain Functions Differ in Rats

Takashi Yamada, Duk-Young Jung, Rumi Sawada, Atsuko Matsuoka,
Ryusuke Nakaoka, and Toshie Tsuchiya*

Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

RESEARCH ARTICLE

Fullerenes are condensed ring aromatic compounds with extended π systems and unique cage structures. Fullerenes are used for medical devices such as carbon nanotubes because they are very flexible and suitable for drug delivery systems. Recently, fullerene derivatives and tube-shaped materials have been used for neuroregeneration studies, and we expect that fullerenes and carbon nanotubes have potential uses as materials in novel medical devices targeting the brain. However, little information on the effects of fullerenes on brain function is available; thus, we examined the effects of [60]fullerene (C_{60}) on the central nervous system in this study. In a V79 cell colony Asia, the IC_{50} of C_{60} was 1620 μg/ml. In an *in vivo* study, 0.25 mg/kg B.W. of C_{60} was injected into the lateral brain ventricle or abdominal cavity of rats. The intracerebral injection of C_{60} increased the locomotor behavior of the rats on days 1 and 30 after the injection. The intraperitoneal injection of C_{60} did not change the locomotor behavior of rats acutely, but it was decreased on day 30. The intracerebral injection of C_{60} affected monoamine concentrations in the rat brain. In particular, serotonin turnover rates were increased in the hypothalamus, cerebral cortex, striatum, and hippocampus, and dopamine turnover rates were increased in the hypothalamus, cerebral cortex, and striatum. The intraperitoneal injection of C_{60} decreased only the dopamine turnover rate in the hippocampus. These results suggest that intracerebral injection of C_{60} had different effects on the central nervous system than intraperitoneal injection. In conclusion, it was suggested that fullerene did not cross the blood-brain barrier. The intracerebral injection of C_{60} affected neurotransmission in the brain widely, and the monoamine dysbolism might be related to changes in locomotor activity.

Keywords: [60]Fullerene, Neurotransmitter, Monoamine, Behavior, Intracerebral Injection.

1. INTRODUCTION

Fullerenes are condensed ring aromatic compounds with extended π systems that have unique cage structures. Recently, fullerenes have been used for medical devices such as carbon nanotubes because they have useful properties for biomedical applications.[1] The biological attributes of fullerenes include photocleavage,[2] antiapoptotic activity,[3] drug and gene delivery,[4,5] antioxidation,[6] chemotaxis,[7] antibacterial activity,[8] and enzyme inhibition.[9] In addition, there are reports of neuroprotective effects of fullerenes. The ability of polyhydroxylated [60]fullerene (C_{60}) to prevent hydrogen peroxide- and cumene hydroperoxide-induced damage in rat hippocampal slices suggested its neuroprotective potency.[10] Hexasulfobutylated [60]fullerene had a neuroprotective effect

on focal cerebral ischemia in rats *in vivo*.[11] These results suggest that fullerenes have neuroprotective effects against oxidative stress. However, examinations of the effects in normal rats and normal condition *in vitro* are few. Tsuchiya et al. evaluated the effects of C_{60} on normal mouse embryonic midbrain cells *in vitro* and mouse embryos *in vivo*. They reported that intraperitoneal (i.p.) injection of C_{60} into pregnant mice at 50 mg/kg exhibited a harmful effect on the embryo head region and tail.[12] It has been suggested that different C_{60} derivatives have different biological actions. Thus, C_{60} derivatives have different cytotoxicity potentials depending on the chemical structure of the derivative, but all C_{60} derivatives have same cage structure and this affects the cytotoxicity of fullerenes.[13] In this study, we used unmodified C_{60} and demonstrated its effects on normal Wistar rats, especially on locomotor behavior and concentrations of brain neurotransmitters.

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*Author to whom correspondence should be addressed.

2. MATERIALS AND METHODS

2.1. V79 Colony Assay

The cytotoxicity of C_{60} was examined by a colony assay using V79 cells as described in the "Guidelines for Basic Biological Tests of Medical Materials and Devices-Part III: Cytotoxicity tests." Chinese hamster fibroblast V79 cells were obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan) and grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a 37 °C humidified atmosphere of 5% CO_2 and 95% air. For the assay, suspensions of 50 V79 cells/ml were seeded on 24-well plates, and the medium was changed to Eagle's MEM supplemented with 5% FBS and 1% penicillin-streptomycin. After 24 incubation in a 37 °C humidified atmosphere of 5% CO_2 and 95% air, 1 ml medium with C_{60} solution (5% C_{60}, 2% Tween 80, 93% sterile water) (Frontier Carbon Co., Tokyo, Japan), 2% Tween 80 without C_{60}, or physiolgical saline solution alone (control) was added to each well, and the cells were cultured for 7 days. The cell cultures were fixed with 10% formalin solution, and the colonies formed were stained with 5% Giemsa solution. The number of colonies in each well was counted, and the efficiency of the culture material was calculated as a ratio of the number of colonies in the sample to that in the control.

2.2. Animals

Male Wistar rats (270 g weight; SLC, Shizuoka, Japan) were kept in individual wire cages in a temperature- and humidity-controlled room (24 °C and 55% relative humidity) under regular lighting conditions (12 h light: dark cycle), and given food and water *ad libitum*. This experiment was carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health Sciences (NIHS).

2.3. Intracerebral and Intraperitoneal Injections of C_{60}

The rats were anesthetized, and a hole was drilled in the skull using a stereotaxic high-speed drill (Muromachi Kikai Co., Ltd., Tokyo, Japan). A microliter syringe was stereotaxically inserted into the right lateral ventricle (0.6 mm posterior to the bregma, 1.6 mm lateral to the midline, and 3.5 mm ventral to the cortical surface) according to the atlas of Paxinos and Watson.[14] C_{60} (0.25 mg/100 g B.W.) dissolved in physiolgical saline solution containing 2% Tween 80 (0.1 μl/100 g B.W.) or physiolgical saline solution containing 2% Tween 80 (0.1 μl/100 g B.W.) was injected at the rate of 1 μl/min using a brain infusion injector (Muromachi Kikai Co., Ltd.). Rats of the control group were injected with the same volume of physiolgical saline solution. The same volume (0.25 mg/100 g B.W.) of C_{60} was

injected intraperitoneally (i.p.) into other rats, and control rats were injected i.p. with the same volume of physiolgical saline.

2.4. Open Field Test

Motor activities (total locomotor distance, average locomotor speed, number of sections crossed, and opening latency) of rats were measured by an open field test. The open field box was 70 × 70 × 70 cm. Tests were performed at 1, 7, and 30 days after brain injection. The rat was placed in the open field box for 10 minutes, and the behavior was recorded using a DV-Track video tracking system CompACT VAS/DV (Muromachi Kikai Co., Ltd.). The floor of the open field box was divided into 16 squares, and one crossing was counted when the rat stepped over a division.

2.5. Neurotransmitter Concentrations in Rat Brain Tissue

After the behavioral test, rats were decapitated, and brain tissues were removed. Wet brain tissue was homogenized in two times the volume of 0.2 M perchloric acid buffer (pH 2) and kept on the ice for 1 hour. Homogenates
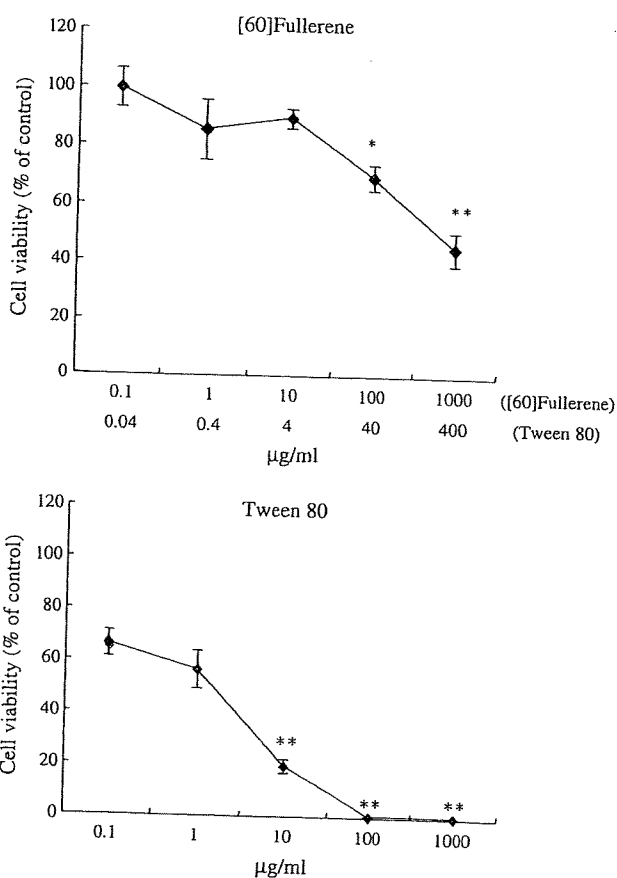

Fig. 1. V79 colony assay. V79 cells were treated with C_{60} and cultured for 7 days. Data are expressed as mean ± SD ($n = 4$). Experimental data showed significant differences from the control group (*; $p < 0.05$ **; $p < 0.01$).

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Table I. Effects of C_{60} and Tween 80 on the survival of rats. C_{60} (0.25 mg/100 g B.W.), Tween 80 (0.1 μl/100 g B.W.), or physiolgical saline (control) was injected into the right lateral ventricle or the abdominal cavity. Surviving rats were counted 24 h after injection.

	Alive (%)	Number of rats
Control	100	(10/10)
[60]Fullerene	100	(8/8)
Tween 80	25	(2/8)

C_{60} (0.25 mg/100 g B.W.), Tween 80 (0.1 μl/100 g B.W.), or physiolgical saline (control) was injected into the right lateral ventricle or the abdominal cavity. Surviving rats were counted 24 h after injection.

were centrifuged at 20,000 g for 15 min at 0 °C, then filtered through a 0.45 μm cellulose acetate membrane filter. Neurotransmitters were detected with an HPLC system under the following conditions: the mobile phase was 0.1 M sodium acetate citric acid buffer and 15% methanol containing EDTA-2Na and 1-octanesulfonic acid sodium salt. The HPLC system was equipped with a reversed-phase column (MA-5ODS, 4.6 × 150 mm; Eicom, Kyoto, Japan) and an electrochemical detector (HTEC-500; Eicom,). Chromatograms were recorded and all calculations were performed using an integrator (EPC-500IS;

Eicom). We measured dopamine (DA), serotonin (5-HT), and norepinephrine (NE), and the metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine hydrochloride (3MT), and 5-hydroxyindole-3-methoxyphenylacetic acid (5HIAA).

3. STATISTICAL ANALYSIS

A Tukey-Kramer test was used to analyze differences in cell viability between the control and the other groups in the V79 colony assay. In the *in vivo* studies, data for the individual groups are expressed as means, and one-way ANOVA was performed for statistical analysis. Student's *t*-test was used to assess differences between the two groups. In all cases, $P < 0.05$ was considered significant. Results are expressed as mean $\pm$ SD.

4. RESULTS

4.1. V79 Colony Assay

Treatment with C_{60} decreased V79 colony formation in a dose-dependent manner. The IC_{50} of C_{60} in V79 cells was
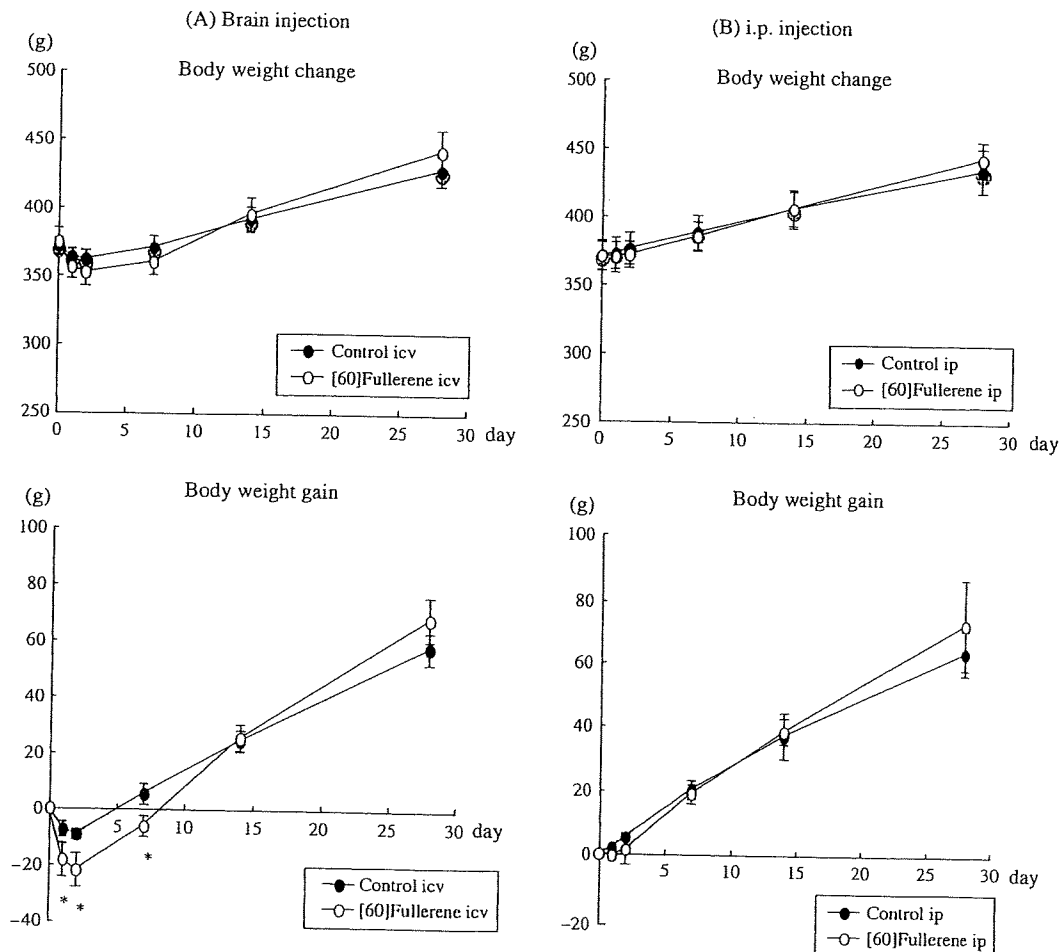
Fig. 2. Body weight change and body weight gain due to C_{60} injection. C_{60} (0.25 mg/100 g B.W.) was injected into the right lateral ventricle (A) or i.p. (B). Control group rats were injected with the same amount of physiolgical saline. Data are expressed as mean $\pm$ SD ($n = 6$). Experimental data showed a significant difference from the control group (*; $p < 0.05$).
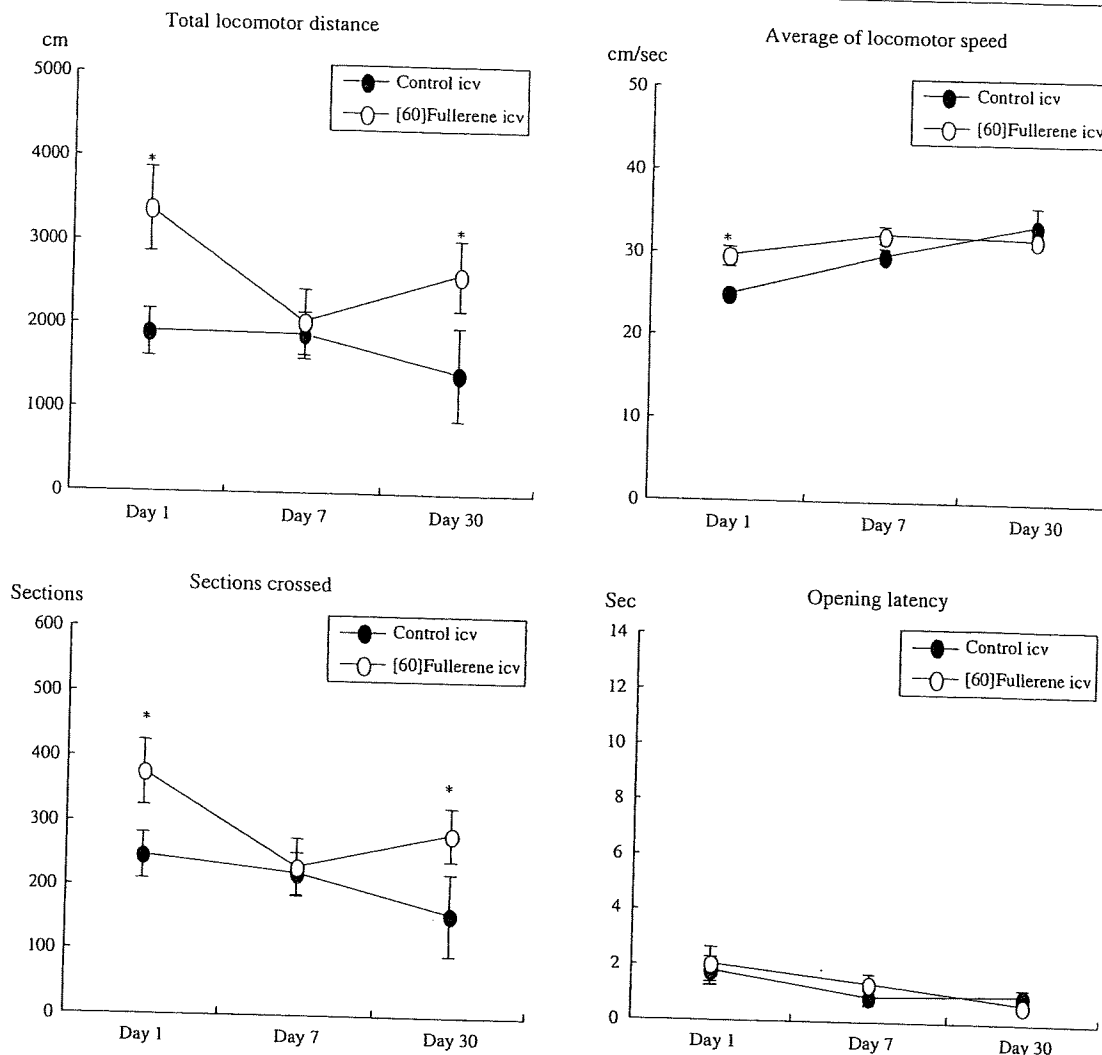
Fig. 3. Effect of intracerebral injection of C_{60} on locomotor activity of rats. C_{60} (0.25 mg/100 g B.W.) was injected into the right lateral ventricle. Control group rats were injected with same amount of physiolgical saline. Open field tests were performed 1, 7, and 30 days after C_{60} brain injection. Data are expressed as mean ± SD ($n = 6$). Experimental data showed significant difference from the control group (*; $p < 0.05$).

1620 μg/ml, and the IC_{50} of Tween 80 in V79 cells was 1.45 μg/ml (Fig. 1). All data are shown as the average of four samples.

4.2. Open Field Test

The intracerebral (i.c.v.) injection of B.W. C_{60} (0.25 mg/100 g containing 0.1 μl Tween 80) did not cause acute toxicity, and all animals survived (Table I). However, injection of Tween 80 (0.1 μl/100 g B.W.) produced acute toxicity, and 75% of the animals died. The i.c.v. injection of C_{60} decreased the postoperative body weight significantly, but the i.p. injection of C_{60} did not change the rat body weight (Fig. 2). In the open field test, the brain C_{60}-injected group ($n = 6$) showed higher total locomotor distance, average locomotor speed, and number of sections crossed compared with control group ($n = 6$) on day 1. On day 7, there was no difference between the two groups. On day 30, total locomotor distance and number

of sections crossed of the intracerebral C_{60}-injected group was increased compared with control group (Fig. 3). In contrast to the brain-injected group, the i.p. C_{60}-injected group had significantly decreased total locomotor distance and number of sections crossed compared with the control group on day 30 (Fig. 4).

4.3. Neurotransmitter Concentrations in Rat Brain Tissue

The intracerebral injection of C_{60} (0.25 mg/100 g containing 0.1 μl Tween 80) decreased NE concentrations in the hypothalamus and hippocampus. Concentrations of serotonin or its metabolite 5HIAA were changed in the hypothalamus, cerebral cortex, striatum, and hippocampus. Concentrations of dopamine or its metabolites DOPAC, HVA, and 3MT were changed in the hypothalamus, cerebral cortex, and striatum. The turnover rates of serotonin were increased in the hypothalamus, cerebral cortex,
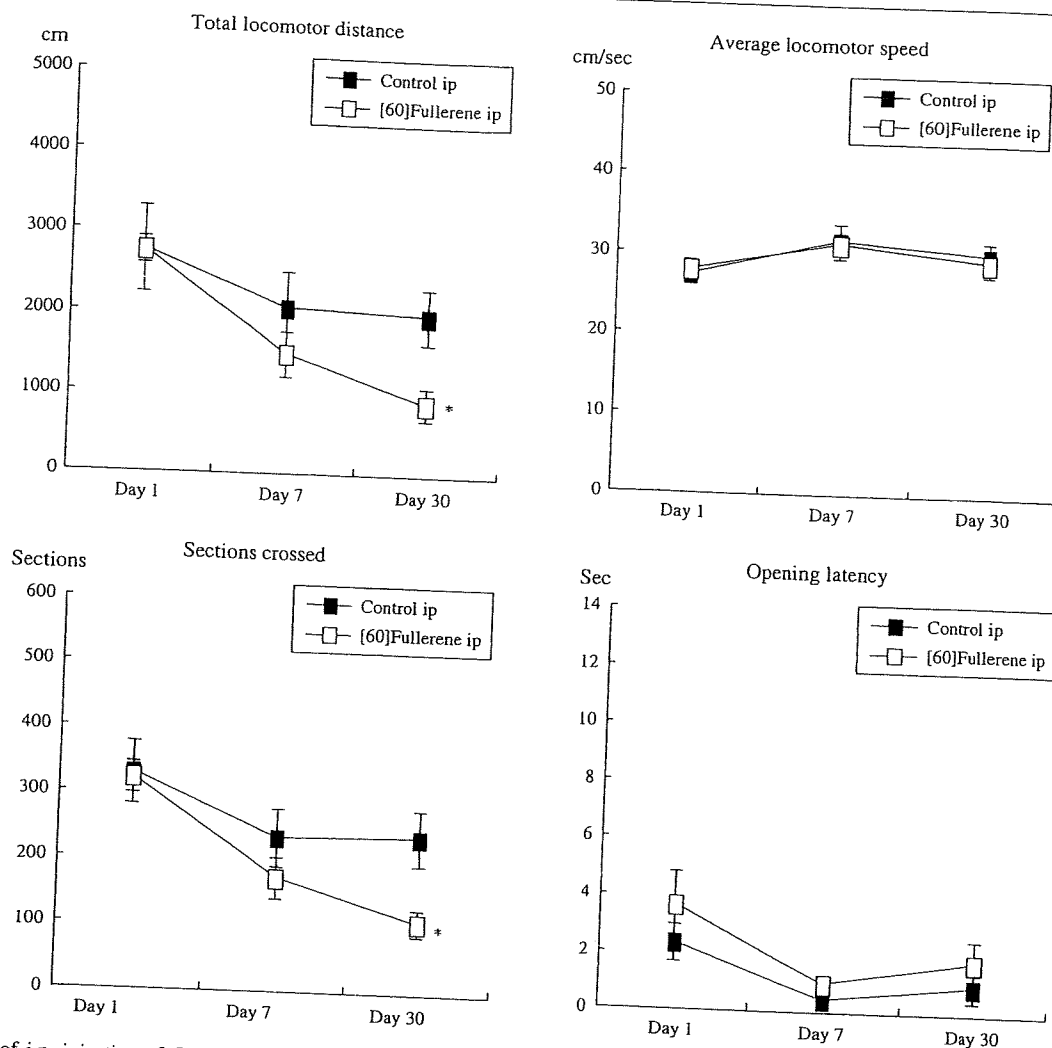
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Fig. 4. Effect of i.p. injection of C_{60} on locomotor activity of rats. C_{60} (0.25 mg/100 g B.W.) was injected into the abdominal cavity. Control group rats were injected with the same amount of physiolgical saline. Open field tests were performed 1, 7, and 30 days after i.p. C_{60} injection. Data are expressed as mean ± SD ($n = 6$). Experimental data showed significant difference from the control group (*; $p < 0.05$).

striatum and hippocampus. The turnover rates of dopamine were increased in the hypothalamus, cerebral cortex, and striatum (Table II). The intraperitoneal injection of C_{60} increased the NE concentration in the hippocampus and the serotonin concentration in the striatum. The turnover rates of dopamine were decreased in the hippocampus. 5HIAA, dopamine, DOPAC, HVA, and 3MT concentrations and the turnover rates of serotonin were not significantly different from the control in any region (Table III).

5. DISCUSSION

NOVEL synthesized biomaterials are attractive candidates for medical devices. In particular, clinical brain and neural treatments, including implantation techniques, have been rapidly advancing, and novel medical devices for the brain are being developed every day. However, adverse events such as foreign-body reaction, inflammation, and tumor formation have been reported in clinical human and animal

studies. In addition, because the blood-brain barrier (BBB) usually protects the central nervous system (CNS) from harmful materials,[15] we consider that the currently available reports about oral or i.p. injection studies and *in vivo* studies are insufficient to establish the effect of the novel materials on brain function. Therefore, investigation of the direct effects of these biomaterials on the CNS before application as medical devices is necessary.

In this study, we demonstrated effects of C_{60} on the CNS and behavior of rats. First, we examined the cytotoxicity of C_{60} and its catalyst Tween 80 by a V79 colony assay. The IC_{50} of Tween 80 in the V79 cells was 1.45 μg/ml. However, IC_{50} of C_{60} in the V79 cells was 1620 μg/ml, and this result indicated that the cytotoxicity of C_{60} on V79 cells is very weak. In this study, C_{60} was dissolved in the culture medium or physiolgical saline solution with 2% Tween 80. Tween 80 is a common detergent, and it has acute cytotoxicity in high doses. Treatment with Tween 80 presented strong cytotoxicity in the V79 colony assay treatment, but C_{60} did not have distinct cytotoxicity in

Table II. Effect of intracerebral C_{60} injection on brain monoamine neurotransmission. C_{60} (0.25 mg/100 g B.W.) was injected into the right lateral ventricle, and rats were decapitated after 30 days. Control group rats were injected with the same amount of physiolgical saline. Data are expressed as mean ± SD ($n = 6$). Experimental data showed a significant difference from the control group (*; $p < 0.05$).

		NE	5HT	5HIAA	DA	DOPAC	HVA	3MT	5HIAA/5HT	(DOPAC+HVA (+3MT))/DA
Hypothalamus	Control	27.51±3.97	9.98±1.38	12.79±1.15	6.21±0.53	2.57±0.23	0.55±0.04		0.91±0.05	0.51±0.03
	[60]fullerene	33.26±2.78	11.68±0.88	16.00±0.88*	7.10±0.64	3.68±0.44*	0.79±0.07*		1.24±0.08*	0.64±0.06*
Cerebral cortex	Control	2.67±0.06	1.35±0.08	1.56±0.08	0.30±0.04	0.14±0.02	0.08±0.01		0.96±0.05	0.57±0.05
	[60]fullerene	2.36±0.14*	1.02±0.06*	1.57±0.12	0.29±0.01	0.20±0.02*	0.11±0.03		1.25±0.08*	0.87±0.01*
Striatum	Control	0.34±0.04	0.59±0.01	1.27±0.04	14.2±0.96	5.62±0.55	1.26±0.10	0.68±0.07	1.77±0.08	0.44±0.02
	[60]fullerene	0.26±0.04	0.54±0.05	1.88±0.25*	18.2±1.86	10.57±1.45*	2.10±0.21*	0.93±0.09*	2.93±0.41*	0.61±0.04*
Hippocampus	Control	2.46±0.08	1.39±0.11	2.25±0.13	0.17±0.02	0.06±0.00	0.03±0.01		1.33±0.04	0.44±0.04
	[60]fullerene	2.08±0.13*	0.87±0.04*	2.00±0.09	0.20±0.02	0.07±0.02	0.04±0.03		1.89±0.07*	0.52±0.28

ng/mg tissue

C_{60} (0.25 mg/100 g B.W.) was injected into the right lateral ventricle, and rats were decapitated after 30 days. Control group rats were injected with the same amount of physiolgical saline. Data are expressed as mean ± SD ($n=6$). Experimental data showed a significant difference from the control group (*; $p < 0.05$).

Table III. Effect of i.p. C_{60} injection on brain monoamine neurotransmission. C_{60} (0.25 mg/100 g B.W.) was injected into the abdominal cavity, and rats were decapitated after 30 days. Control group rats were injected with the same amount of physiolgical saline. Data are expressed as mean ± SD ($n = 6$). Experimental data showed significant difference from the control group (*; $p < 0.05$).

		NE	5HT	5HIAA	DA	DOPAC	HVA	3MT	5HIAA/5HT	(DOPAC+HVA (+3MT))/DA
Hypothalamus	Control	31.93±2.08	14.07±0.89	12.09±1.72	5.74±0.75	3.58±0.23	0.97±0.18		1.23±0.10	0.83±0.10
	[60]fullerene	32.98±1.89	13.18±1.22	18.73±2.32	6.19±0.33	3.70±0.51	0.75±0.51		1.62±0.18	0.71±0.05
Cerebral cortex	Control	2.60±0.19	1.21±0.06	1.70±0.16	0.31±0.02	0.17±0.01	0.07±0.01		1.15±0.07	0.65±0.08
	[60]fullerene	2.75±0.17	1.25±0.09	1.98±0.14	0.29±0.03	0.21±0.03	0.09±0.02		1.30±0.08	0.81±0.06
Striatum	Control	0.41±0.14	0.58±0.05	1.31±0.07	16.5±0.58	8.10±0.31	1.64±0.06	0.76±0.03	1.86±0.09	0.52±0.03
	[60]fullerene	0.31±0.05	0.87±0.09*	1.47±0.10	15.5±1.28	8.37±0.49	1.62±0.11	0.71±0.04	1.49±0.25	0.57±0.02
Hippocampus	Control	2.09±0.17	1.16±0.11	2.28±0.21	0.10±0.03	0.08±0.01	0.19±0.01		1.61±0.10	2.62±0.37
	[60]fullerene	2.49±0.15*	1.08±0.07	2.20±0.10	0.19±0.04	0.11±0.04	0.16±0.03		1.67±0.04	1.31±0.27*

tissue ng/mg

C_{60} (0.25 mg/100 g B.W.) was injected into the abdominal cavity, and rats were decapitated after 30 days. Control group rats were injected with the same amount of physiolgical saline. Data are expressed as mean ± SD ($n = 6$). Experimental data showed significant difference from the control group (*; $p < 0.05$).

the *in vitro* study. In addition, intracerebral injection of 0.1 μl/100 g B.W. Tween 80 alone caused acute toxicity and death of injected rats, but intracerebral injection of C_{60} with 0.1 μl/100 g B.W. Tween 80 into the rat brain and intraperitoneal injection did not produce acute toxicity *in vivo*. These results suggest that C_{60} prevented the acute cytotoxicity of Tween 80. Some reports have shown that C_{60} has a cytoprotective effect against some toxicants and oxidative stress *in vitro* and *in vivo*, and we assumed that Tween 80 does not remain long-term *in vivo*.[16, 17] These results suggest that the co-injection of C_{60} and Tween 80 did not have cytotoxicity or that it was very weak in this study.

Intracerebral injection of C_{60} decreased the postoperative body weight of rats, but i.p. injection did not. The body weight loss was not fatal, but the result suggested that brain injection of C_{60} decreased food intake. However, in the open field test, total locomotor distance, locomotor speed, and numbers of sections crossed were increased significantly by C_{60} on the day after operation. In addition, the body weight gain of C_{60} injected rats tended to have increased more than that of control rats by day 30. These results suggest that brain injection of C_{60} affected neurotransmission-related feeding behavior. Thus, we measured serotonin (5HT) and norepinephrine (NE), and the metabolite 5HIAA. 5HT and NE are found abundantly in the hypothalamus, which is the main source of neurotransmission. 5HT and NE neurotransmissions relate to feeding, emotion, emotional behavior, stress, hormone secretions; dysbolisms of these neurotransmitters cause cibophobia, hyperphagia, dystrophy, or depression-like behavior.[18, 19] Intracerebral injection of C_{60} changed the serotonin and 5HIAA concentrations in the hypothalamus, cerebral cortex, striatum, and hippocampus, and 5HT turnover rates were increased in these four regions. The results suggest that C_{60} increased 5HT neurotransmission. NE concentrations also changed in the cerebral cortex and hippocampus. It is known that 5HT and NE neurotransmissions are enhanced when homeostasis is in disorder, for example, due to stress or inflammation and a variety of other changes in peripheral conditions.[18, 19] However, i.p. injection of C_{60} increased the 5HT concentration only in the striatum, and did not affect 5HT turnover rates in the entire region. These results suggest that C_{60} did not pass the BBB to affect brain neurotransmission directly. On the other hand, some substances, for example, the glutamate receptor agonist kainic acid,[20] the gamma-aminobutyric acid (GABA) receptor antagonist strychnine,[21] and other neurotoxins[22] express neurotoxicity at lower doses than the C_{60} concentration used in this study and cause vital body weight loss and locomotor difficulty or death. These results suggested that intracerebral injection of C_{60} did not cause acute harmful neurotoxicity, but it did cause inflammation or neurotransmission disability of monoamines in the brain. The intracerebral injection of C_{60} also increased

dopamine turnover rates in the hypothalamus, cerebral cortex, and striatum. Dopamine is an important neurotransmitter for behavior in the brain striatum, and dopamine neurotransmission promoted ambulation or decreased anxiety in several rat studies.[23, 24] Dopamine dysbolism is related to depression, and depletion of dopamine causes Parkinson's disease.[25, 26] Infusion of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a dopaminergic neuron-specific neurotoxin, into the brain reduced cognitive and motor functions in the rat and decreased dopamine concentration in the prefrontal cortex and striatum.[27] Rats that exhibit depressive-like behavior due to intraperitoneal administration of the kappa-opinion receptor agonist salvinorin A had reduced extracellular concentrations of dopamine within the nucleus accumbens.[28] In contrast, an excess of dopamine neurotransmission induces locomotor hyperactivity.[29, 30] Dopaminergic neurotransmission is closely involved in motor activity and emotional behavior. In this study, we showed an increase of dopamine turnover rates that was consistent with the increase of locomotion on day 30. On the other hand, i.p. injection of C_{60} did not change concentrations of dopamine and its metabolites in all brain regions examined or the dopamine turnover rate in the striatum. These results suggest that brain injection of C_{60} increased dopamine neurotransmission similar to serotonin in the brain and that the dopamine neurotransmission might induce locomotor hyperactivity behaviors.

In conclusion, we investigated the neurotoxicity of intracerebral injection of C_{60} and compared it with i.p. injection of C_{60}. In the *in vitro* study, we showed that the cytotoxicity of C_{60} was about 1620 μg/ml (10 ppm) in V79 cells. In an *in vivo* study, intracerebral injection of 0.25 mg/ml C_{60} did not cause acute vital neurotoxicity. However, some neurotransmitter concentrations were changed in several brain regions. We suggest that chronic expose of the brain to C_{60} may cause serotoninergic and dopaminergic neurotransmission abnormalities and change behavior and homeostasis. Recently, medical progress had produced novel medical devices targeting the brain. We recommend reexamination of the materials used for medical devices targeting on brain.

References and Notes

1. D. Pantarotto, N. Tagmatarchis, A. Bianco, and M. Prato, *Mini Rev. Med. Chem.* 4, 805 (2004).
2. K. Irie, Y. Nakamura, H. Ohigashi, H. Tokuyama, S. Yamago, and E. Nakamura, *Biosci. Biotechnol. Biochem.* 60, 1359 (1996).

3. L. L. Dugan, J. K. Gabrielsen, S. P. Yu, T. S. Lin, and D. W. Choi, *Neurobiol. Dis.* 3, 129 (1996).
4. T. Y. Zakharian, A. Seryshev, B. Sitharaman, B. E. Gilbert, V. Knight, and L. J. Wilson, *J. Am. Chem. Soc.* 127, 12508 (2005).
5. H. Isobe, W. Nakanishi, N. Tomita, S. Jinno, H. Okayama, and E. Nakamura, *Chem. Asian. J.* 17, 167 (2006).
6. M. Bisaglia, B. Natalini, R. Pellicciari, E. Straface, W. Molorni, D. Monti, C. Franceschi, and G. Schettini, *J. Neurochem.* 74, 1197 (2000).
7. C. Toniolo, A. Bianco, M. Maggini, G. Scorrano, M. Prato, M. Marastoni, R. Tomatis, S. Spisani, G. Palu, and E. D. Blair, *J. Med. Chem.* 37, 4558 (1994).
8. S. Bosi, T. Da Ros, S. Castellano, E. Banfi, and M. Prato, *Bioorg. Med. Chem. Lett.* 10, 1043 (2000).
9. S. Marchesan, T. Da Ros, G. Spalluta, J. Balzarini, and M. Prato, *Bioorg. Med. Chem. Lett.* 15, 3615 (2005).
10. M. C. Tsai, Y. H. Chen, L. Y. Chiang, and H. C. Huang, *J. Pharm. Pharmacol.* 49, 438 (1997).
11. S. S. Huang, S. K. Tsai, C. L. Chih, L. Y. Chiang, H. M. Hsieh, C. M. Teng, and M. C. Tsai, *Free Radic. Biol. Med.* 30, 634 (2001).
12. T. Tsuchiya, I. Oguri, Y. Yamakoshi-Nakajima, and N. Miyata, *FEBS Lett.* 393, 139 (1996).
13. C. M. Sayes, J. D. Fortner, W. Guo, D. Lyon, A. M. Boyd, K. D. Ausman, Y. J. Tao, B. Sitharaman, L. J. Wilson, J. B. Hughes, J. L. West, and V. L. Colvin, *Nano Lett.* 4, 1881 (2004).
14. G. Paxinos and C. Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press, New York (1986).
15. R. A. Yokel, *J. Alzheimer's Dis.* 10, 223 (2006).
16. L. Xiao, H. Takada, X. Gan, and N. Miwa, *Bioorg. Med. Chem. Lett.* 16, 1590 (2006).
17. N. Gharbi, M. Pressac, M. Hadchouel, H. Szwarc, S. R. Wilson, and F. Moussa, *Nano Lett.* 5, 2578 (2005).
18. E. B. Olson and W. P. Morgan, *Life Sci.* 30, 2095 (1982).
19. S. Checkley, *Pharmacopsychiatry* 21, 6 (1988).
20. J. V. Nadler, *Adv. Biochem. Psychopharmacol.* 27, 395 (1981).
21. M. C. Anderson, E. Chung, and M. H. Van Woert, *Eur. J. Pharmacol.* 155, 279 (1988).
22. T. Narahashi, J. M. Frey, J. S. Ginsburg, and M. L. Roy, *Toxical. Lett.* 64–65, 429 (1992).
23. W. Schultz, *Trends Neurosci.* 30, 203 (2007).
24. D. P. Taylor, L. A. Riblet, H. C. Stanton, A. S. Eison, and D. L. Temple, Jr., *Pharmacol. Biochem. Behav.* 17, 25 (1982).
25. W. T. Blows, *J. Neurosci. Nurs.* 32, 234 (2000).
26. A. Tessitore, R. A. Hariri, F. Fera, G. W. Smith, N. T. Chase, M. T. Hyde, R. D. Weinderger, and S. V. Mattay, *J. Neurosci.* 22, 9099 (2002).
27. R. D. Prediger, L. C. Batista, R. Medeiros, P. Pandolfo, J. C. Florio, and R. N. Takahashi, *Exp. Neurol.* 202, 391 (2006).
28. W. A. Carlezon, C. Begin, J. A. DiNieri, M. H. Baumann, M. R. Richards, M. S. Todtenkopf, R. B. Rothman, Z. Ma, D. Y. Lee, and B. M. Cohen, *J. Pharmacol. Exp. Ther.* 16, 440 (2006).
29. G. Biala, *Postepy. Hig. Med. Dosw.* 58, 560 (2004).
30. D. Viggiano, D. Vallone, and A. Sadile, *Neural. Plast.* 11, 97 (2004).

RESEARCH ARTICLE

Intracerebral Microinjection of Stannous 2-Ethylhexanoate Affects Dopamine Turnover in Cerebral Cortex and Locomotor Activity in Rats

Takashi Yamada, Duk-Young Jung, Rumi Sawada, Toshie Tsuchiya

Division of Medical Devices, National Institute of Health Sciences, Tokyo, Japan

Abstract: Stannous 2-ethylhexanoate [$Sn(Oct)_2$] is used as a catalyst for production of poly-L-lactic acid and copolymers that are implanted in cranial surgery, but reports on its effects on the central nervous system are few. We examined the effects of $Sn(Oct)_2$ on cell viability *in vitro* and on neurotransmission and behavior in the rat. Treatment of normal human astrocytes with 10 mg/mL $Sn(Oct)_2$ reduced mitochondrial activity to 16% of the control. Injection of $Sn(Oct)_2$ at 6.28 mg/kg BW (2 mg/kg BW Sn) into right lateral ventricle of the rat brain tended to increase the ambulation distance after 30 days when compared with the control group. The turnover of dopamine neurotransmission was increased in the cerebral cortex. These results suggest that $Sn(Oct)_2$ is cytotoxic to astrocytes *in vitro*. Injection of $Sn(Oct)_2$ into the brain had no or very weak immediate neurotoxicity, but long-term exposure to $Sn(Oct)_2$ increased dopamine neurotransmission turnover. © 2008 Wiley Periodicals, Inc.
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Keywords: stannous 2-ethylhexanoate; open field test; dopamine, serotonin, cytotoxicity

INTRODUCTION

Biodegradable polymers are attractive candidates for scaffolding materials because they degrade as new tissues are formed. However, adverse events, such as foreign-body reaction, inflammation, and tumor formation, have been reported in human clinical and animal studies. Therefore, confirmation that these polymers and related materials used as catalysts lack toxicity is required.

Stannous 2-ethylhexanoate [$Sn(Oct)_2$] is commonly used as a catalyst in the production of poly-L-lactic acid (PLLA) and other copolymers that are implanted in cranial surgery.[1] Food dishes and trays are also made of PLLA. The toxicity of $Sn(Oct)_2$ has been examined in *in vitro* and *in vivo* studies.[2,3] $Sn(Oct)_2$ is resolved into Sn and 2-ethylhexanoic acid in the stomach.[4] Therefore, the toxicity of $Sn(Oct)_2$ depends on the toxicities of Sn and 2-ethylhexanoic acid.[5] It is known that 2-ethylhexanoic acid is metabolized from 2-ethylhexanol in the body and that the acceptable daily intake (ADI) of 2-ethylhexanoic acid is 0.5 mg/kg BW/day.[6,7] The provisional tolerable weekly intake (PTWI) of tin is 14 mg/kg BW/week.[8,9]

$Sn(Oct)_2$ has recently been used as a catalyst in the production of medical devices. In these cases, $Sn(Oct)_2$ does not degrade to Sn and 2-ethylhexanoic acid because it does not pass through the acidic conditions of the stomach. However, little information about the effects of the combination of Sn and 2-ethylhexanoic acid on mammals is available. It was reported that a combination of harmful materials increases the materials' toxicity.[10] Based on these reports, we considered that further examination is required before $Sn(Oct)_2$ is used as a catalyst in the production of medical device materials.

Brain and clinical neurology sciences are advancing rapidly, and implantation techniques and novel medical devices for the brain are being developed every day. A synthetic biodegradable dura mater has recently been developed using PLLA and a copolymer of polyglycolic acid or polycaprolactic acid as the major components. The blood–brain barrier normally protects the central nervous system (CNS) from harmful materials,[11,12] but implantation of the synthetic dura mater directly exposes the CNS to unknown conditions and necessitates investigation of the direct effects of biomaterials used as medical devices on the CNS.

In this study, we examined the effects of $Sn(Oct)_2$ on cell viability in normal human astrocytes (NHA) *in vitro* and the effects of direct injection of $Sn(Oct)_2$ into the rat brain on neurotransmitters and behavior *in vivo*.

MATERIALS AND METHODS

Astrocyte Microtiter Tetrazolium Assay

The effects of $Sn(Oct)_2$ on mitochondrial activity of NHA were measured using a microtiter tetrazolium (MTT) assay. NHA were seeded into 24-well plates at a density of 1×10^4/well in ABM medium supplemented with 5% FBS and recombinant human epidermal growth factor, insulin, GA-1000, ascorbic acid, L-glutamate (ANG bred kit; Sanko Junyaku, Tokyo, Japan) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. $Sn(Oct)_2$ were added directly to the ABM medium. After 1-week culture with 2.5, 5, or 10 μg/mL of $Sn(Oct)_2$, the medium in each well was replaced with 300 mL of fresh medium containing 6 mL TetraColor ONE reagent (Seikagaku, Tokyo, Japan). After 2 h, the absorbance at 450/630 nm was measured using a plate reader. The data were expressed as averages of five wells, and the procedures were performed in triplicate.

Animals

Male Wistar rats (270 g weight; SLC, Shizuoka, Japan) were kept in individual wire cages in a temperature- and humidity-controlled room (24°C and 55% relative humidity) under regular light conditions (12 h light:dark cycle) and given food and water *ad libitum*. Six rats were used in the experimental group, and six rats were used as controls. This experiment was carried out in accordance with the guidelines for the care and use of laboratory animals of the National Institutes of Health Sciences, which refer to standards of the American Association for Laboratory Animal Science.

Intracerebral Injection of Sn(Oct)₂

Rats were anesthetized, and a hole was drilled in the skull using a high-speed drill (Muromachi Kikai, Tokyo, Japan). A microliter syringe was stereotaxically inserted into the right lateral ventricle (0.6 mm posterior to the bregma, 1.6 mm lateral to the midline, and 3.5 mm ventral to the cortical surface) according to the atlas of Paxinos and Watson,[13] and 6.28 mg/kg BW $Sn(Oct)_2$ (2 mg/kg BW tin) was injected at the rate of 1 mL/min, using a brain infusion stereotaxic injector (Muromachi Kikai, Tokyo, Japan). Control rats were injected with the same volume of Ringer's solution.

Open Field Test

Motor activities (total locomotion distance, average locomotion speed, number of sections crossed, and beginning latency) of rats were measured by an open field test. The open field box was 70 cm $\times$ 70 cm $\times$ 70 cm. Tests were performed 1, 7, and 30 days after $Sn(Oct)_2$ injection. The rat was placed in the open field box, and its behavior was recorded using a DV-Track video tracking system CompACT VAS/DV (Muromachi Kikai, Tokyo, Japan). The floor of the open field box was divided into 16 squares, and one crossing was counted when the rat stepped over the divisions.

Neurotransmitter Concentrations in Brain Tissue

After the last behavioral test, the rat was decapitated, and brain tissues were removed. Wet brain tissues were homogenized in two volumes of 0.2M perchloric acid buffer (pH 2) and kept on ice for 1 h. Homogenates were centrifuged at 20,000g for 15 min at 0°C, then filtered through a 0.45 μm cellulose acetate membrane filter. Neurotransmitters were detected with an HPLC system under the following conditions: the mobile phase was 0.1M sodium acetate citric acid buffer and 15% methanol containing EDTA-2Na and 1-octanesulfonic acid sodium salt. The HPLC system was equipped with a reversed-phase column (MA-5ODS, 4.6 $\times$ 150 mm^2: Eicom, Kyoto, Japan) and an electrochemical detector (HTEC-500: Eicom, Kyoto, Japan). Recording of chromatograms and all calculations were performed using an integrator (EPC-500IS: Eicom, Kyoto, Japan). We measured dopamine (DA), serotonin (5-HT), and norepinephrine (NE), and their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine hydrochloride, and 5-hydroxyindole-3-methoxyphenylacetic acid (5HIAA).

Statistical Analysis

The Tukey–Kramer test was used to analyze differences in cell viability between the control group and the test groups in the NHA MTT assay. *In vivo* data were expressed as means for individual groups, and one-way ANOVA was performed for statistical analysis. Student's t-test was used to assess differences between the two groups. In all cases, $p < 0.05$ was considered significant. Results were expressed as mean $\pm$ SD.

RESULTS

Astrocyte Microtiter Tetrazolium Assay

Treatment of $Sn(Oct)_2$ decreased NHA mitochondrial activity in a dose-dependent manner (Figure 1). Treatment with 10 mg/mL $Sn(Oct)_2$ decreased NHA mitochondrial activity to 16% of the control. All data shown were the average of five samples.

Open Field Test

The injection of 6.28 mg/kg BW $Sn(Oct)_2$ (2 mg/kg BW tin) into the brain did not significantly change the average body weight, but did reduce the body weight gain (Figure 2). In the open field test, the total locomotion distance and average locomotion speed were decreased in the rats ($n = 6$) injected with $Sn(Oct)_2$, and the beginning latency was
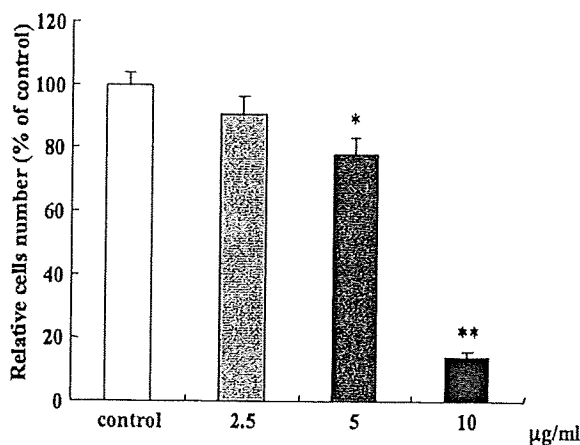
Figure 1. The effect of $Sn(Oct)_2$ on astrocytes. Astrocytes were cultured with 10 mg/mL $Sn(Oct)_2$ for 7 days, and mitochondrial activity of NHA was measured by MTT assay. Data are expressed as mean ± SD (n = 5). Experimental data showed significant difference from the control group (*$p < 0.05$; **$p < 0.01$).

increased compared with the control group (n = 6) on day 1. On day 7, there was no difference between the two groups. On day 30, the total locomotion distance and crossing sections tended to increase in the $Sn(Oct)_2$-injected group, and the beginning latency was decreased. Ambulation decreased in the control group with the repetition of tests, in contrast with the $Sn(Oct)_2$-injected group, in which it increased (Figure 3).

Neurotransmitters in Rat Brain Tissue

Injection of $Sn(Oct)_2$ into the rat brain decreased NE concentrations in the rat hypothalamus (Table I) and decreased the DA concentration in the cerebral cortex. Concentrations of DOPAC and HVA, which are DA metabolites, and the turnover rate of DA were increased in the cerebral cortex (Table I). Concentrations of 5HT and its metabolite 5HIAA were increased in the striatum by $Sn(Oct)_2$ injection. In the hippocampus, the turnover rate of 5HT was increased by $Sn(Oct)_2$ injection.

DISCUSSION

There are many reports of synthesis of novel polymers and biomaterials using $Sn(Oct)_2$ as a catalyst, but few studies of the toxicity of $Sn(Oct)_2$. $Sn(Oct)_2$ is used as a catalyst in food dish production, but its safety has been examined only in the case of oral consumption. Almost all $Sn(Oct)_2$ is resolved into Sn and 2-ethylhexanoic acid in the stomach.[4] It is known that 2-ethylhexanoic acid is metabolized from 2-ethylhexanol in the body and that the ADI for rats is 0.5 mg/kg BW/day.[6,7] The PTWI for humans of tin is 14 mg/kg BW/week (~2 mg/kg BW/day).[8,9] However, the effects of injection of $Sn(Oct)_2$ into the brain have not been examined.

In this study, we demonstrated the effects of $Sn(Oct)_2$ on the CNS and behavior of rats. Treatment of NHA

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in vitro with 10 mg/mL $Sn(Oct)_2$ decreased mitochondrial activity by 16% when compared with the control. It was suggested that the reduction of mitochondrial activity decreases ATP production. ATP not only drives energy-dependent reactions inside cells, but also functions as a potent signaling molecule in the CNS. Therefore, this result suggests that $Sn(Oct)_2$ is cytotoxic to CNS cells[14] and that specific verification of the safety of $Sn(Oct)_2$ is required before it is used as a catalyst in the synthesis of medical devices, especially in the cases of biodegradable and bioabsorbable polymers.

Intracranial injection of $Sn(Oct)_2$ did not decrease the body weight of rats, but it did decrease the weight gain. In the open field test, total locomotion distance and locomotion speed decreased, and beginning latency was increased due to $Sn(Oct)_2$ injection on day 1. On day 30, in contrast to day 1, the $Sn(Oct)_2$-injected group tended to exhibit increased total locomotion distance and crossing frequency and decreased beginning latency. Ambulation decreased in
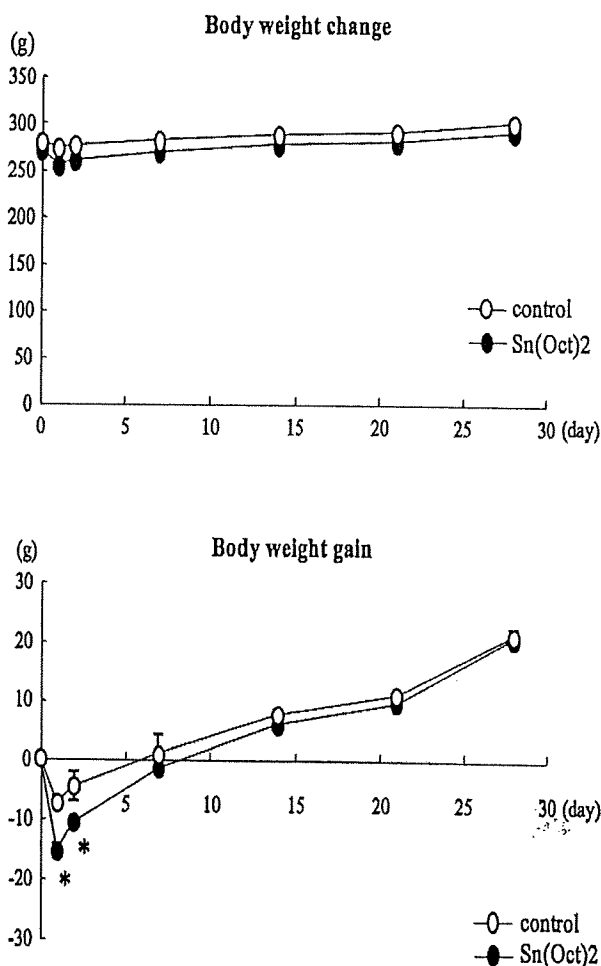

Figure 2. Changes in body weight and body weight gain because of intracranial injection of $Sn(Oct)_2$. $Sn(Oct)_2$ at 6.28 mg/kg BW (2 mg/kg BW tin) was injected into the right lateral ventricle. Control group rats were injected with the same amount of Ringer's solution. Data are expressed as mean ± SD (n = 6). *Experimental data showed a significant difference from the value of day 0 ($p < 0.05$).
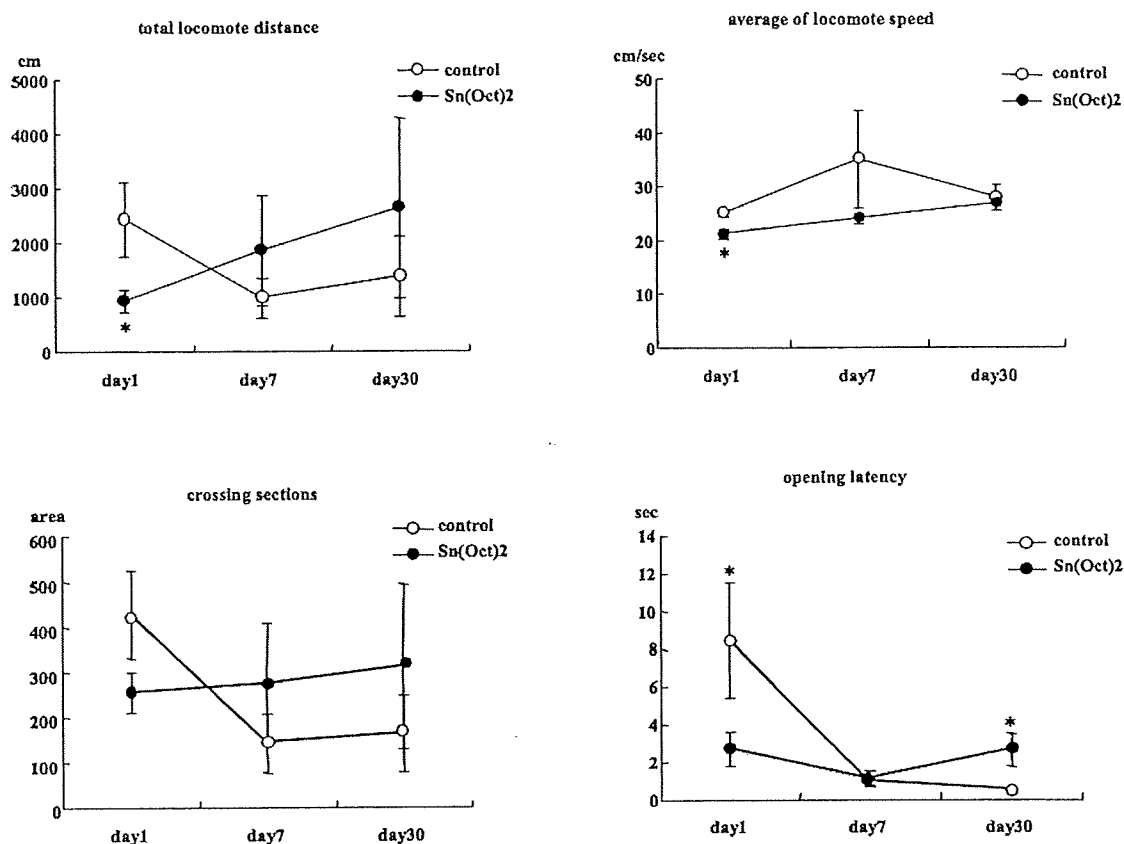
Figure 3. Effect of $Sn(Oct)_2$ on locomotion in rat. $Sn(Oct)_2$ at 6.28 mg/kg BW (2 mg/kg BW tin) was injected into the right lateral ventricle. Control rats were injected with the same amount of Ringer's solution. Open field tests were performed 1, 7, and 30 days after $Sn(Oct)_2$ injection. Data are expressed as mean ± SD (n = 6). *Experimental data showed a significant difference from the control group ($p < 0.05$).

the control group with experience in performing the test, and, in contrast, ambulation by the $Sn(Oct)_2$-injected group increased. This result suggests that intracranial injection of $Sn(Oct)_2$ decreased locomotor activity immediately but increased it afterwards. Some substances, for example, the glutamate receptor agonist kainic acid,[15] the GABA receptor antagonist strychnine,[16] and other neurotoxins[17] produce neurotoxicity at doses lower than the $Sn(Oct)_2$ concentration used in this study and cause body weight loss and locomotive difficulty or death. $Sn(Oct)_2$ did not have acute neurotoxicity or it was very weak at the $Sn(Oct)_2$ concentration used in this study. However, locomotion distance was increased by $Sn(Oct)_2$ injection after 30 days. Generally, locomotion distance in the open field test is decreased by repeated testing, and the results of our previous tests corresponded to this observation.[18] Thus, we suspect that $Sn(Oct)_2$ might produce chronic neurotoxicity.

Because intracranial injection of $Sn(Oct)_2$ tended to increase locomotion distance in repeat tests, we measured brain monoamines of $Sn(Oct)_2$-injected rats. The brain monoamines DA, 5-HT, and NE are major neurotransmitters related to emotion, stress, and several other functions.[19,20] DA is an important neurotransmitter for behavior. DA neurotransmission promotes ambulation and

decreases anxiety in the rat.[21,22] DA dysfunction is related to depression, and DA depletion causes Parkinson's disease.[23,24] Infusion of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, which is a dopaminergic neuron-specific neurotoxin, reduced the cognitive and motor functions of the rat and decreased DA concentration in the prefrontal cortex and striatum.[25] Administration of the kappa-opioid receptor agonist salvinorin A to depressed rats reduced the extracellular concentration of DA within the nucleus accumbens.[26] Therefore, dopaminergic neurotransmission is closely involved in motor activity and emotional behavior. In this study, intracranial injection of $Sn(Oct)_2$ decreased the DA concentration in the cerebral cortex and increased concentrations of the DA metabolites DOPAC and HVA significantly. These results mean that $Sn(Oct)_2$ enhanced DA transmission and turnover. The increase of DA turnover due to $Sn(Oct)_2$ was consistent with the increase in locomotion distance observed in this study. On the other hand, the injection of $Sn(Oct)_2$ into the brain decreased the NE concentration in the rat hypothalamus, and increased concentrations of 5HT and its metabolite 5HIAA in the striatum. In the hippocampus, the rate of 5HT turnover was increased by $Sn(Oct)_2$ injection. NE and 5HT neurotransmissions are related to emotion, emotional behavior, stress,

TABLE I. Effect of Sn(Oct)₂ on Brain Monoamine Neurotransmission and 5HT and DA Turnover Rates

		NE	5HT	5HIAA	DA	DOPAC	HVA	3MT	5HIAA/5HT	(DOPAC+HVA(+3MT))/DA
Hypothalamus	control	78.0 ± 3.0	22.3 ± 0.9	26.9 ± 3.0	8.46 ± 0.79	6.82 ± 1.09	0.80 ± 0.06		1.12 ± 0.08	0.55 ± 0.06
	Sn(Oct)2	70.6 ± 2.3ᵃ	19.2 ± 1.6	27.1 ± 4.5	7.76 ± 0.66	7.98 ± 1.15	0.97 ± 0.09		1.30 ± 0.06	0.71 ± 0.03
Cerebral cortex	control	2.78 ± 0.08	1.48 ± 0.14	1.85 ± 0.14	0.21 ± 0.01	0.09 ± 0.01	0.06 ± 0.00		1.19 ± 0.09	0.52 ± 0.09
	Sn(Oct)2	2.55 ± 0.14	1.48 ± 0.08	2.12 ± 0.08	0.16 ± 0.01ᵃ	0.20 ± 0.05ᵃ	0.11 ± 0.03ᵃ		1.34 ± 0.15	1.34 ± 0.35ᵃ
Striatum	control	0.52 ± 0.05	1.21 ± 0.07	1.70 ± 0.09	19.7 ± 1.02	7.85 ± 0.62	1.58 ± 0.07	0.79 ± 0.07	1.34 ± 0.12	0.34 ± 0.03
	Sn(Oct)2	0.56 ± 0.09	1.53 ± 0.13ᵃ	2.12 ± 0.15ᵃ	19.3 ± 1.82	8.90 ± 1.11	1.87 ± 0.18	0.81 ± 0.08	1.30 ± 0.03	0.39 ± 0.03
Hippocampus	control	19.5 ± 0.74	5.57 ± 0.23	6.71 ± 0.74	2.12 ± 0.20	1.70 ± 0.27	0.20 ± 0.02		1.36 ± 0.10	0.50 ± 0.02
	Sn(Oct)2	17.7 ± 0.57	4.79 ± 0.41	6.78 ± 1.12	1.94 ± 0.16	1.99 ± 0.29	0.24 ± 0.02		1.69 ± 0.13ᵃ	0.64 ± 0.07

Sn(Oct)₂ at 6.28 mg /kg B.W. (2 mg/kg B.W. tin) was injected into the right lateral ventricle of rats, and control rats were injected same amount of Ringer's solution. Rats were decapitated after 30 days. Dates are expressed as mean ± SD (n=6).
ᵃ Experimental data showed a significant difference from control group data ($p < 0.05$).

and hormone secretion, and dysbolisms of these neurotransmitters can cause dystrophy or depression-like behavior.[27,28] Based on these results, we suggest that intracranial injection of Sn(Oct)₂ changed the neurotransmission of DA and other monoamines and that it affected the motor activity of rats. In this study, we measured DA and concentrations of its metabolites only 30 days after Sn(Oct)₂ injection, and all *in vivo* studies were performed after injection of 6.28 mg/kg BW Sn(Oct)₂ [2 mg/kg BW (2 ppm) tin]. However, these results should be compared with the clinical findings. Therefore, follow-up studies are needed to examine the effects of different concentrations of Sn(Oct)₂, injection times, and observation periods. In addition, it is necessary to estimate the concentration of tin degraded from a medical prosthesis, such as a dura mater.

CONCLUSIONS

We investigated the neurotoxicity of Sn(Oct)₂. The *in vitro* study demonstrated that Sn(Oct)₂ is cytotoxic to NHA at 10 mg/mL (10 ppm). The *in vivo* study showed that injection of 6.28 mg/kg BW Sn(Oct)₂ [2 mg/kg BW (2 ppm) tin] into rat brain did not produce remarkable acute neurotoxicity. However, we found for the first time that Sn(Oct)₂ caused dopaminergic neurotransmission abnormalities and altered locomotive behavior for 30 days after a single intracerebral microinjection. Recent medical progress has produced novel medical devices targeting the brain, but we recommend re-examination of medical devices targeting the brain that use these types of catalysts.

REFERENCES

1. Tsuji H, Nishikawa M, Sakamoto Y, Itsuno S. Novel preparation method for poly(L-lactide)-based block copolymers: Extended chain crystallites as a solid-state macro-coinitiator. Biomacromolecules 2007;8:1730–1738.
2. Ikarashi Y, Tsuchiya T, Nakamura A. Effect of heat treatment of poly(L-lactide) on the response of osteoblast-like MC3T3-E1 cells. Biomaterials 2000;12:1259–1267.
3. Ahmed S, Tsuchiya T. A mouse strain difference in tumorigenesis induced by biodegradable polymers. J Biomed Mater Res A 2006;79:409–417.
4. Penninks AH, Hilgers L, Seinen W. The absorption, tissue distribution and excretion of di-*n*-octyltin dicholoride in rats. Toxicology 1987;44:107–120.
5. Moody DE, Reddy JK. Serum triglyceride and cholesterol contents in male rats receiving diets containing plasticizers and analogues of the ester 2-ethylhexanol. Toxicol Lett 1982;10:379–383.
6. Albro PW. The metabolism of 2-ethylhexanol in rats. Xenobiotica 1975;5:625–636.
7. Badr MZ, Handler JA, Whittaker M, Kauffman FC, Thurman RG. Interactions between plasticizers and fatty acid metabolism in the perfused rat liver and in vitro. Inhibition of ketogenesis by 2-ethylhexanol. Biochem Pharmacol 1990;39:715–721.
8. Noel L, Leblanc JC, Guerin T. Determination of several elements in duplicate meals from catering establishments using closed vessel microwave digestion with inductively coupled

plasma mass spectrometry detection: Estimation of daily dietary intake. Food Addit Contam 2003;20:44–56.

9. Ysart G, Miller P, Croasdale M, Crews H, Robb P, Baxter M, de L'Argy C, Harrison N. 1997 UK Total Diet Study—Dietary exposures to aluminium, arsenic, cadmium, chromium, copper, lead, mercury, nickel, selenium, tin and zinc. Food Addit Contam 2000;17:775–786.

10. Jonker D, Woutersen RA, van Bladeren PJ, Til HP, Feron VJ. Four-week oral toxicity study of a combination of eight chemicals in rats: Comparison with the toxicity of the individual compounds. Food Chem Toxicol 1990;28:623–631.

11. Yokel RA. Blood–brain barrier flux of aluminum, manganese, iron and other metals suspected to contribute to metal-induced neurodegeneration. J Alzheimers Dis 2006;10:223–253.

12. Zheng W. Neurotoxicology of the brain barrier system: New implications. J Toxcol 2001;9:711–719.

13. Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. New York: Academic Press; 1986.

14. Volonte C, Amadio S, Cavaliere F, D'Ambrosi N, Vacca F, Bernardi G. Extracellular ATP and neurodegeneration. Curr Drug Targets CNS Neurol Disord 2003;2:403–412.

15. Nadler JV. Role of excitatory pathways in the hippocampal damage produced by kainic acid. Adv Biochem Psychopharmacol 1981;27:395–402.

16. Anderson MC, Chung E, Van Woert MH. Strychnine seizure potentiation by azaspirodecanedione anxiolytics in rats. Eur J Pharmacol 1988;155:279–283.

17. Narahashi T, Frey JM, Ginsburg JS, Roy ML. Sodium and GABA-activated channels as the targets of pyrethroids and cyclodienes. Toxical Lett 1992;64/65:429–436.

18. Bouwknecht JA, Spiga F, Staub DR, Hale MW, Shekhar A, Lowry CA. Differential effects of exposure to low-light or high-light open-field on anxiety-related behavior: Relationship to c-Fos expression in serotonergic and non-serotonergic neurons in the dorsal raphe nucleus. Brain Res Bull 2007;72:32–43.

19. Liu Y, Nakamura S. Stress-induced plasticity of monoamine axons. Front Biosci 2006;11:1794–1801.

20. Chaney DS. Monoamine dysfunction and the pathophysiology and treatment of depression. J Clin Psychiatry 1998;59:11–14.

21. Schultz W. Behavioral dopamine signals. Trends Neurosci 2007;30:203–210.

22. Taylor DP, Riblet LA, Stanton HC, Eison AS, Temple DL. Dopamine and antianxiety activity. Pharmacol Biochem Behav 1982;17:25–35.

23. Blows WT. Neurotransmitters of the brain: Serotonin, noradrenaline (norepinephrine), and dopamine. J Neurosci Nurs 2000;32:234–238.

24. Tessitore A, Hariri RA, Fera F, Smith GW, Chase NT, Hyde MT, Weinderger RD, Mattay SV. Dopamine modulates the response of the human amygdala: A study in Parkinson's disease. J Neurosci 2002;22:9099–9103.

25. Prediger RD, Batista LC, Medeiros R, Pandolfo P, Florio JC, Takahashi R. The risk is in the air: Intranasal administration of MPTP to rats reproducing clinical features of Parkinson's disease. Exp Neurol 2006;202:391–403.

26. Carlezon WA, Begin C, DiNieri JA, Baumann MH, Richards MR, Todtenkopf MS, Rothman RB, Ma Z, Lee DY, Cohen BM. Depressive-like effects of the κ-opioid receptor agonist salvinorin A on behavior and neurochemistry in rats. J Pharmacol Exp Ther 2006;316:440–447.

27. Olson EB, Morgan WP. Rat brain monoamine levels related to behavioral assessment. Life Sci 1982;30:2095–2100.

28. Checkley S. Monoamine, depression and antidepressant drugs. Pharmacopsychiatry 1988;21:6–8.
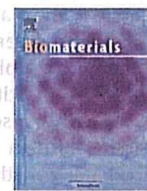
The effect of sulfated hyaluronan on the morphological transformation and activity of cultured human astrocytes

Takashi Yamada, Rumi Sawada, Toshie Tsuchiya*

Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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ABSTRACT

We demonstrated the effect of synthesized sulfated hyaluronan (SHya), which is composed of a sulfated group and hyaluronan, and basic fibroblast growth factor 2 (FGF-2) on normal human astrocytes (NHA) activity and its morphological transformation in vitro study. Astrocyte is a kind of glial cell and stellated astrocyte (activating astrocyte) supports axons network, neurons survival and synaptic plasticity. Treatment of SHya hardly affected NHA proliferation. However combination treatment of SHya and FGF-2 increased NHA proliferation. Treatment of SHya promoted transformation of normal astrocyte into a stella morphology (stellation) and combination treatment of SHya and FGF-2 promoted stellation than that of SHya only. Treatment of SHya increased glial fibrillary acidic protein (GFAP), nestin mRNA and GFAP protein expression in the stellated NHA. The cell–cell adhesion of NHA increased by treatment of SHya. Treatment of SHya increased heparin-binding trophic factors FGF-2, midkine, and some other trophic factors mRNA level in the NHA. These results suggested that the treatment of SHya promoted NHA activity due to enhancing neurotrophins production and the morphological transformation of NHA and the effect of SHya on astrocytes partly involved FGF-2 activity. These findings indicate that SHya may be involved in the astrocyte activity and support neurons survivals.

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1. Introduction

The central nervous system (CNS) comprises neurocytes and glial cells. Recent studies have suggested that glial cells play important roles in neuroprotection and neurogenesis [1–3]. Glial cells not only sustain the neuron network physically but also supply neurotrophic factors to neurons, modify neurotransmission, and support neuroregeneration. Astrocytes, a type of glial cell, intercede between neurons and blood vessels and form the blood–brain barrier. Astrocytes protect axons from injury and promote neuron plasticity by producing trophic factors, such as basic fibroblast growth factor 2 (FGF-2) and glia cell line-derived neurotrophic factor (GDNF) [4,5]. Moreover, astrocytes contribute to neural stem cell (NSC) proliferation and differentiation and the plasticity of neurons. Especially in the region of an injury, normal resting astrocytes transform themselves into activated astrocytes to perform their functions. Activated astrocytes themselves, which have a stellar morphology, increase immunohistochemical reactions of intermediate proteins such as glial fibrillary acidic protein (GFAP) and express the NSC marker nestin, and stellation enhances the production of neurotrophic factors by astrocytes [6,7]. In addition,

activation of astrocytes increases the production of FGF-2, midkine, nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1), brain-derived neurotrophic factor (BDNF), and other trophic factors that contribute to the survival and neurogenesis of neurons and NSC. These results suggest that the activation of astrocytes to supply neurotrophic factors may be an effective therapy for central nervous diseases [4,8] and repair of brain injury.

Glucosaminoglycans have one or more sulfate groups, which have negative charge, and these sulfate groups attract growth factors such as FGF-2 and midkine. Several glucosaminoglycans contribute to the activities of neurotrophic factors. Heparin, a sulfated glucosaminoglycan, binds FGF-2 and enhances FGF-2 bioactivity in the central nervous system [9,10]. A recent study reported that chondroitin sulfate, an oligosaccharide that has sulfate groups, combines with midkine and BDNF to promote neuronal growth in cultured hippocampal neurons [11]. These pleiotrophin-/heparin-binding growth-associated molecules, FGF and midkine, contribute to survival and proliferation of immature and mature central neurocytes and astrocytes. FGF-2 is involved in survival of immature neural progenitor cells, and FGF-2 (20 ng/ml) and epidermal growth factor (EGF) (20 ng/ml) maintained neural stem cell proliferation in vitro [12]. FGF-2 also supported the survival and proliferation of transplanted progenitor cells in the adult rat striatum [13]. It was reported that hippocampal cells are generated over the lifetime of an animal and that FGF-2 stimulates proliferation of these cells to

* Corresponding author. Tel./fax: +81 3 3700 1487.
  E-mail address: tsuchiya@nihs.go.jp (T. Tsuchiya).