cycle was observed. The grain count in 24-hr autoradiographs was at the background level. Fig. 2 shows the distribution of grain count in the testis on 6-hr and 24-hr autoradiographs.

2. Electron microscopic autoradiography

In either 6-hr or 24-hr autoradiographs, no grains were observed in any specimens of testes, liver, or kidneys by electron microscopy.

DISCUSSION

In the present study, a single dose of DEHP (1000 mg/kg), labeled with ^{3}H at the phthalic acid or at the

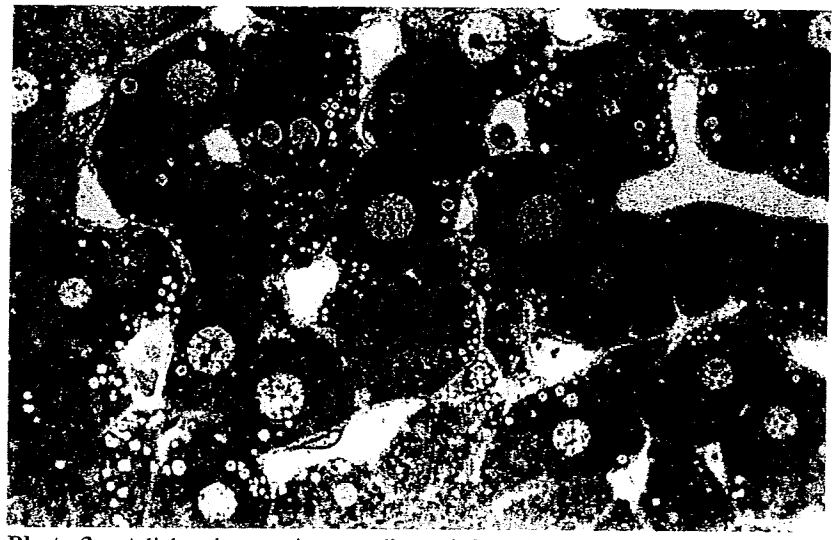


Photo 3. A light microscopic autoradiograph from the liver of a rat sacrificed 24 hr after administration of [3,4,5,6-^{3}H]-phthalic acid di(2-ethylhexyl)ester. A few grains are noted on the hepatocytes, × 800.

Photo 4. A light microscopic autoradiograph from the kidney of a rat sacrificed 6 hr after administration of [3,4,5,6-^{3}H]-phthalic acid di(2-ethylhexyl)ester. Many silver grains are noted at the luminal border of epithelial cells of the straight part of proximal tubule, × 800.

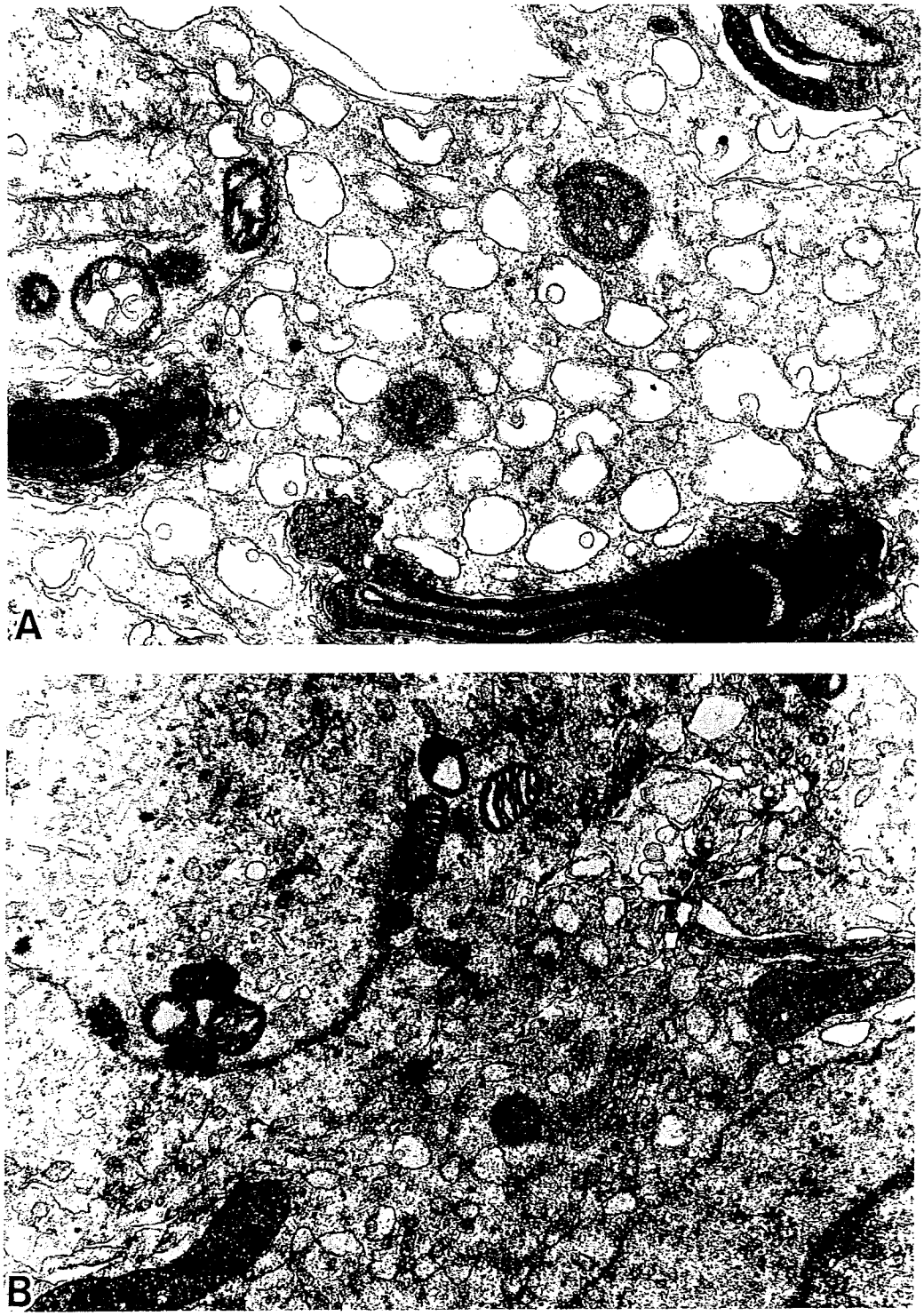


Photo 5. Electron microscopic autoradiographs from seminiferous tubules of testis sacrificed 6 hr after administration of [3,4,5,6-^{3}H]-phthalic acid di(2-ethylhexyl) ester.
A: A few silver grains are noted on the mitochondria and smooth-surfaced endoplasmic reticulum of Sertoli cell, × 22800.
B: A few silver grains are noted on the extracellular space. Seminiferous tubule at stage I of spermatogenesis, × 17100.

Table 1. Distribution of silver grains on electron microscopic autoradiographs after single oral administration of DEHP.

Group	Organ Cell	Organelle	After administration 6 hr	 24 hr
[3,4,5,6-^{3}H]-Phthalic acid, di-(2-ethylhexyl)ester : DEHP-^{3}H	Testis			
	Sertoli cell	mitochondria	••••••	••
		s-ER	••••••••••••	••••••••
		r-ER		••••
		extracellular space	•••	••••••••••••••
		Golgi apparatus	•	
		lysosome	•	
	spermatogonia			••••••••
	spermatocyte		•••	••••••••
	spermatid			•••
	Liver			
	hepatocyte	mitochondria	••	••••••••••••
		r-ER	••••	•••••
		peroxisome	••••	••
	sinusoid			
	Kidney			
	proximal tubule	brush border	••••••••••	•
	epithelial cell	mitochondria	••••	•••••
		s-ER	••••	
		lysosome		••
		peroxisome	•••	
		Golgi apparatus	•	
Phthalic acid, di-(2-ethyl[1^{3}H]hexyl)ester : ^{3}H-DEHP			ND	ND

ND: not detected, •: count of silver grains.

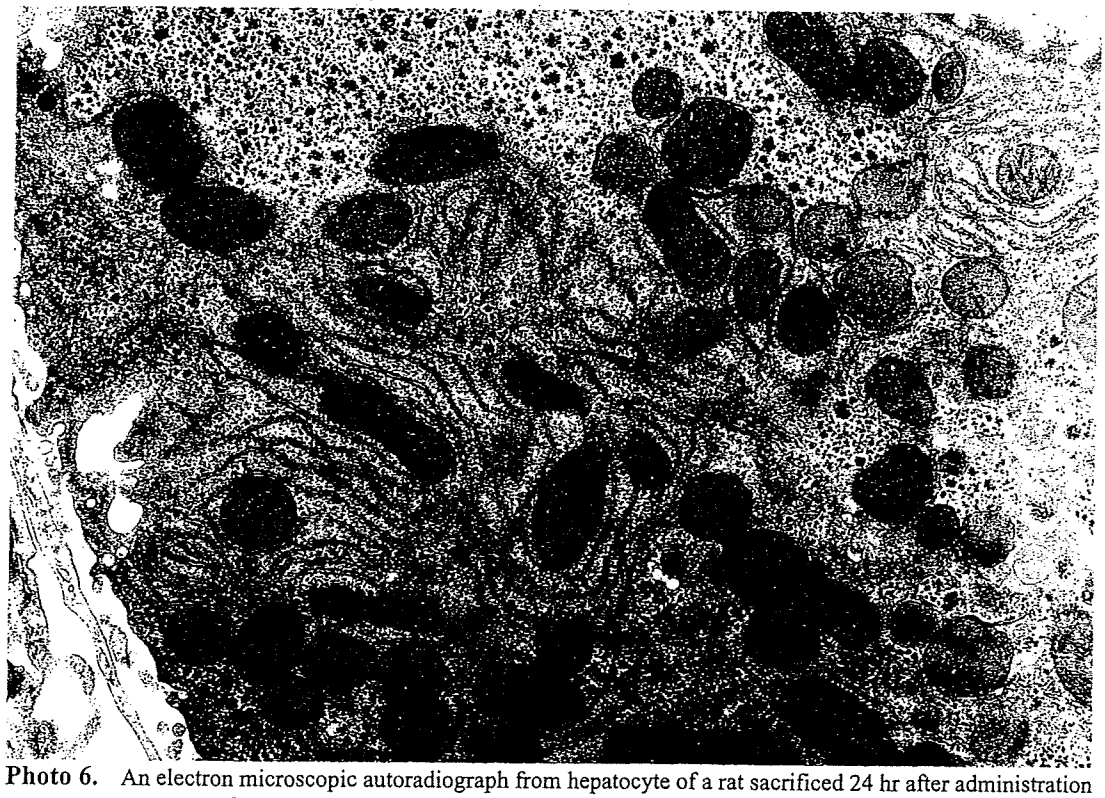


Photo 6. An electron microscopic autoradiograph from hepatocyte of a rat sacrificed 24 hr after administration of [3,4,5,6-^{3}H]-phthalic acid di(2-ethylhexyl)ester. A few silver grains are noted on the mitochondria, rough-surfaced endoplasmic reticulum and peroxisome., × 14000.

alcohol was administered to 8-week-old rats of the Sprague-Dawley strain (92.5 MBq/rat). The rats were sacrificed and autopsied at 6 or 24 hr after administration, autoradiographs prepared for the testes, liver, and kidneys, and observed under light microscope or electron microscope.

On the autoradiographs of animals administered with DEHP-^{3}H, labeled at the phthalic acid, considerable distribution of radioactivity was observed in the testes at the basal area of seminiferous tubules at the stages from IX to I of spermatogenic cycle, which corresponded well to the location of DEHP toxicity observed in our previous study (Saitoh *et al.*, 1997). By electron microscopic observation, subcellular localization of radio-sensitized grains was observed at smooth-surfaced endoplasmic reticulum and mitochondria of Sertoli cells. Fewer grains were also noted at the Golgi apparatus and lysosome of Sertoli cells, and at the junctions between the neighboring Sertoli cells or between a Sertoli cell and spermatocytes. On the other hand, on the autoradiographs by administration of ^{3}H-DEHP, labeled at the alcohol moiety, only a few grains were observed by light microscopy in the testes at 6 hr after administration.

There are already many studies on the distribution of DEHP in the body. Schulz and Rubin (1973) studied the metabolism of ^{14}C-DEHP by oral administration in rats and reported that more than 80% of radioactivity was recovered within 24 hr, about 35% in urine and 55% in feces. Williams and Blanchfield (1974) administered ^{14}C-DEHP orally to rats, and observed that most of the radioactivity was excreted in urine and feces by 24 hr after administration and that the distribution of radioactivity among tissues was most highly to the spleen, testes, and adipose tissue, followed by kidneys and liver. Radioactivity levels in tissues reached the peak 4 hr after administration, and no radioactivity was observed 24 hr after administration. On the other hand, the study on oral administration of ^{14}C-DEHP by Daniel (1978) suggested that enterohepatic circulation of DEHP or its metabolites might have occurred, since 14% of radioactivity was excreted in bile for 4 days. Gaunt and Butterworth (1982) have conducted a study of whole-body autorad-

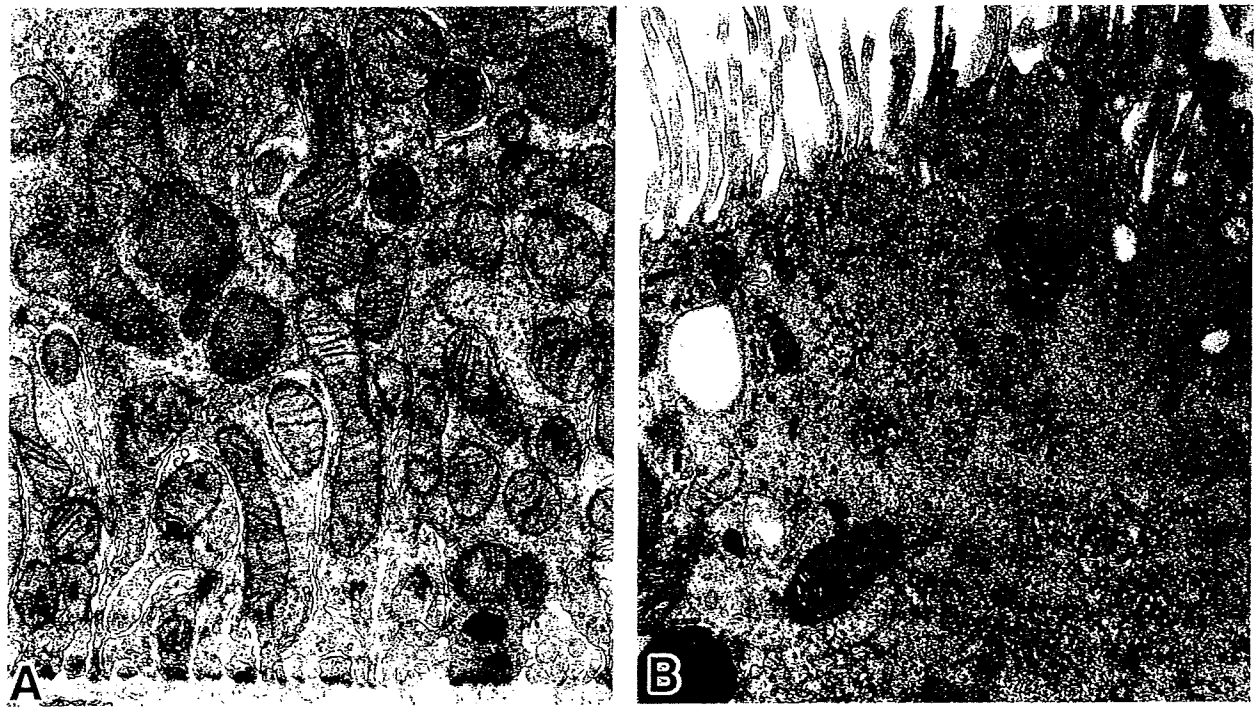


Photo 7. Electron microscopic autoradiographs from renal tubular epithelial cell of a rat sacrificed 6 hr after administration of [3,4,5,6-^{3}H]-phthalic acid di(2-ethylhexyl)ester. A few silver grains are noted on the brush border, smooth surfaced endoplasmic reticulum and mitochondria of the cell.
A: Basal area, × 10640.
B: Adluminal area, × 12160.

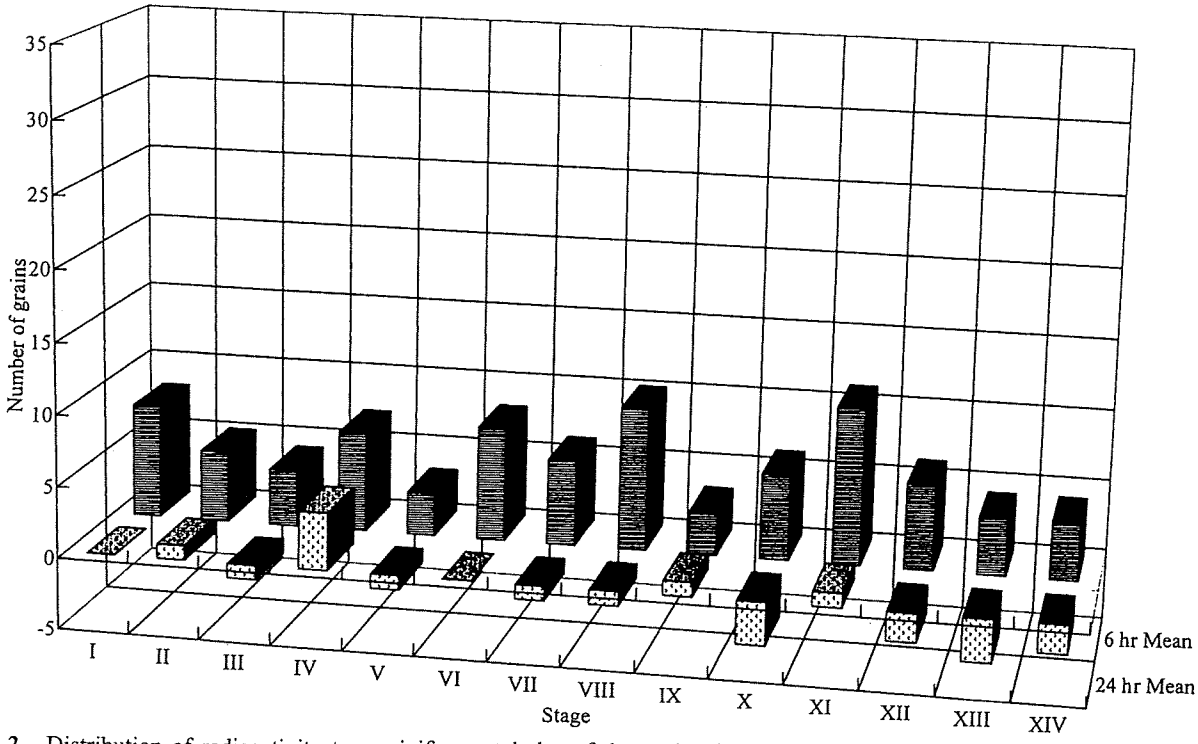
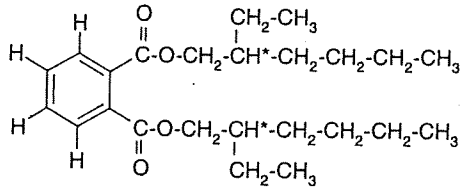
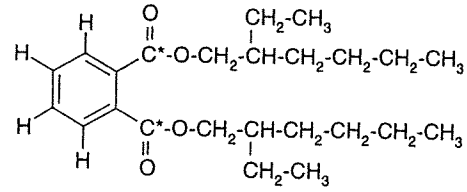


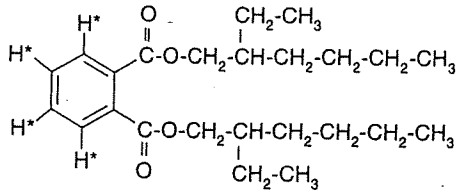
Fig. 2. Distribution of radioactivity to seminiferous tubules of the testis of rats 6 and 24 hr after administration of di(2-ethyl[1-^{3}H]hexyl)ester. Mean counts of radiosensitized grains per seminiferous tubule on the autoradiographs are shown by the stage of spermatogenesis.



Phthalic acid, di (2-ethyl [1-^{3}H] hexyl)ester
: [1-^{3}H] DEHP



[7-^{14}C]-Phthalic acid di (2-ethylhexyl)ester
: [7-^{14}C] DEHP



[3,4,5,6-^{3}H] Phthalic acid di (2-ethylhexyl)ester
: [3,4,5,6-^{3}H] DEHP

Fig. 3. Chemical structures of the labeled compounds used in the present study and used in the study of Gray and Gangolli (1986). The positions of radio-labeling are shown with *.

iography in mice by oral administration of ^{14}C-DEHP and observed that radioactivity levels of the intestinal wall, renal pelvis, bladder, urine, and liver reached their peaks at 4 hr after administration, and that they decreased afterwards except in the renal pelvis. In the present study, distribution of radioactivity observed as grains in testes and kidneys at 6 hr after administration of DEHP-^{3}H, significantly decreased at 24 hr after administration. Thus, DEHP or its metabolites distributed to testes and kidneys after single administration disappeared from the tissue rather fast. On the other hand, the grain count of the liver at 24 hr increased more than at 6 hr after administration, which suggests that DEHP or its metabolites were accumulated in the liver, possibly by enterohepatic circulation.

On autoradiography with oral administration of ^{3}H-DEHP, only a few radiosensitized grains were observed under light microscopic observation of thick sections of any tissues of the testes, liver or kidneys, and no grains were observed on electron microscopic autoradiography. This result implies that DEHP is rapidly splitted in the body into phthalic acid and alcohol, and only the phthalic acid moiety is transported into the tissues. It has been reported that orally administered phthalate diesters such as DEHP are rapidly changed in the gut to monoesters such as MEHP and absorbed, and that MEHP was similarly as potent as DEHP in causing testicular changes (Gray and Gangolli, 1986; Thomas and Thomas, 1984). On the other hand it has been reported that oral administration of phthalic acid did not cause testicular atrophy in rats (Cater *et al.*, 1977). From this and other experimental evidence, MEHP has been assumed to be the proximate toxicant (Albro *et al.*, 1989). In the present study, the radioactivity of DEHP labeled at the phthalic acid moiety penetrated the blood-testis barrier into Sertoli cells, while almost no tissue distribution of radioactivity were observed by autoradiography with DEHP labeled at the alcohol moiety (Fig. 1 and 2). Since MEHP metabolized from ^{3}H-DEHP was still radioactive, it is considered that orally administered DEHP was metabolized to MEHP before intestinal absorption and then further metabolized to the form of phthalic acid and taken into the testicular tissue. Thus, the phthalic acid moiety seems to be responsible for toxicological changes in testes. However, contradictory to the present results, Gray and Gangolli (1986) reported that ^{14}C-MEHP penetrated the blood-testis barrier only to a very limited extent. They used DEHP labeled with ^{14}C supposedly at the 7th position of phthalic acid or carbonyl carbon (Fig. 3). (They have not described the labeled position of the compound, but preceding papers had given the relevant information (Albro *et al.*, 1973; Schultz and Rubin, 1973).) It is chemically implausible that the carbonyl radical is detached so easily from the phthalic acid in the body. The conclusion of Gray and Gangolli (1986) that MEHP did not pass the blood-testis barrier was drawn from their observation that very little ^{14}C-MEHP appeared in rete testis fluid 25 min after intravenous administration. They remarked that MEHP affected Sertoli cells from the outside of the cells. The present study has demonstrated with autoradiography that the radioactivity of DEHP labeled at the phthalic acid moiety did pass the blood-testis barrier into Sertoli cells. Differences of test methods, amount of radioactivity used and timing of measurement may explain the discrepancy.

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Comparative Studies on Levels of Proteins, Bacterial Endotoxins and Nucleic Acids in Hyaluronan Preparations Used to Treat Osteoarthritis of the Knee

—Could Residual Proteins and Bacterial Endotoxins Relate to Complications?—

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Comparative Studies on Levels of Proteins, Bacterial Endotoxins and Nucleic Acids in Hyaluronan Preparations Used to Treat Osteoarthritis of the Knee

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ABSTRACT

The levels of proteins, bacterial endotoxins and absorbance of 260nm related to deoxyribonucleic acids (DNA) were measured in several hyaluronan (HA) preparations used for the treatment of knee osteoarthritis (OA) : Hyalgan®, Artz®† and Synvisc®. Hyalgan® and Artz® are classified as non-modified HA preparations, while Synvisc® is classified as a modified HA preparation in which the HA chains are cross-linked, utilizing proteins derived from chicken combs. Therefore, Synvisc® was included relatively large amount of residual proteins in comparison with that of other HA preparations. The residual protein contents in Hyalgan®, Artz® and Synvisc® were 4.5 ± 0.6 μg/mL, 0.5 ± 0.1 μg/mL and 7.5 ± 1.8 μg/mL, respectively.

The contaminant levels of bacterial endotoxins in Hyalgan®, Artz® and Synvisc® were 0.0074 ± 0.0026 EU/mL, 0.0045 ± 0.0001 EU/mL and 0.19915 ± 0.0741 EU/mL.

Spectrophotometric analyses (absorbance at 260nm) in Hyalgan®, Artz® and Synvisc® were 0.107 ± 0.004, 0.026 ± 0.003 and 0.167 ± 0.073.

These results indicated that Synvisc® included quite high content of parameters such as bacterial endotoxins, proteins in comparison with non-modified HA preparation ; Hyalgan® and Artz®. (**Jpn Pharmacol Ther 2004 ; 32 : 655-62**)

KEY WORDS Hyaluronan preparation, Protein, Bacterial endotoxin, Deoxyribonucleic acid, Knee osteoarthritis, Inflammation

Introduction

Since the late 1980's, the intraarticular injection of hyaluronan (HA) has been recognized as a useful treatment for the symptoms of knee osteoarthritis (OA)[1~3]. Several HA products that differ in their molecular weights (**Table 1**)[4, 21] have been developed for this purpose. To summarize, the HA in all of these preparations were

Hatano Research Institute, Food and Drug Safety Center

† Artz® is one of the trade names, which include Artz®, Artzal® and Supartz®.

Table 1 General properties of hyaluronan preparations used in this study[4,21]

Test preparation	Active substance	Molecular weight	Complex viscosity	% Elasticity	Concentration of active substance	Clinical dosage schedule
		$\times 10^6$ Dalton	Pa·sec at 0.02 Hz	at 3 Hz	mg/mL	mL×no. of weeks
Hyalgan®	Non-modified hyaluronan	0.5−0.73	< 0.1	29	10	2.0×5
Artz®	Non-modified hyaluronan	0.62−1.17	0.3	33	10	2.5×5
Synvisc®	Modified hyaluronan	> 6	213	88	8	2.0×3

extracted from chicken combs ; the average approximate molecular weight, in daltons, of the HA in each product is 0.50-0.73 million in Hyalgan®, 0.62-1.17 million in Artz®, and over 6.0 million in Synvisc®. The active substance in Hyalgan® and Artz® are non-modified HA, which have lower molecular weight than the HA found in joint fluid (whose molecular weight is 4-6 million daltons). Although the non-modified HA in these preparations clears more rapidly from the joint cavity than does the "modified" HA of Synvisc®, it is considered that non-modified HA might remain active after permeation into the cartilage and synovial membrane. For example, the design concept of Artz®, termed "Joint Fluid Therapy", is intended to improve not only the lubricative property of the knee joint but also the metabolism of the cartilage or synovial membrane[5,6].

On the other hand, the active substance of Synvisc®, called Hylan, is prepared by cross-linking, thus modifying, HA molecules. Synvisc® is composed of 80% Hylan A fluid and 20% Hylan B gel. Hylan A is prepared by bridging between two or more HA molecules using formaldehyde and proteins derived from chicken combs[7]. Hylan B is prepared by forming cross-links between the hydroxyl groups of the Hylan A or HA using sulfonyl-bis-ethyl[7]. The design concept of Synvisc®, described as "Viscosupplementation", is based on the prolonged retention in the joint cavity of the modified HA, as compared to that of the non-modified HA preparations, which maintains a long-lasting heightened viscoelasticity of the joint fluid[4,7].

Recently, HA injection into a joint as a therapy for knee OA have become widely used throughout the world and is placed a common therapy in Japan. Safety profile of patients in Artz® has been indicated low frequency of adverse event (0.50% ; 37 cases in 7,404 cases) by the post market surveillance for 6 years[8]. Although it appears that all HA preparations present a favorable safety profile[9], severe acute inflammatory reactions (SAIRs)[10,11] and pseudogout[12] have been reported as complications occurring after the intraarticular injection of certain HA preparations for the treatment of knee OA, even though these injections were conducted under strictly aseptic conditions. Therefore, it is possible that some of these cases may be caused by the HA therapy itself. For example, Puttick et al. reported that acute local inflammatory reactions were observed in 27% of patients treated with intraarticular injections of Synvisc®[10]. Although the mechanisms of these acute inflammatory reactions are still unclear, there is a possibility that they were induced by foreign proteins and other contaminants in the HA preparation.

In general, the contaminant levels of proteins, bacterial endotoxins and nucleic acids (DNA) are common indicators in process control of productions and purification process of the preparation. We measured those parameters in several HA preparations in this study.

Materials and Methods

1 Test preparations

Hyalgan® (Lots No. 059100, 057800, 061600 ; manufactured by FIDIA SpA, Italy), Artz® (Lots No. 1F379D, 1G381D, 1G382D ; manufactured by Seikagaku Corporation, Japan) and Synvisc® (Lots No. Q0009, Y9914, Z9906 ; manufactured by Biomatrix, USA) were used in this study.

2 Reagents

Folin-Ciocalteu reagent [2-fold dilution of Phenol Reagent, Wako Pure Chemical Industries, Ltd. Japan] was used for the determination of protein levels. Toxicolor system LS-50M set (Lot No. 34M055 ; Seikagaku Corporation) and Toxicolor system DIA set (Lot No. 510232 ; Seikagaku Corporation) were used as reagents for the determination of bacterial endotoxin levels. Endotoxin 100 (Control No. 0103, National Institute of Health Sciences, Japan) was used as a standard endotoxin.

3 Determination of protein levels

The determination of protein levels in each preparation was performed according to the Lowry method[13]. A 500 mg sample of each test preparation was weighed, mixed with an alkaline copper test solution, and then the Folin-Ciocalteu reagent was added. The absorbance of the resultant solution at 750 nm was measured using a spectrophotometer (UVIDEC-610, Japan Spectroscopic Co. Ltd. Japan). The protein content of each preparation was calculated from the calibration curve constructed from the results of a standard solution prepared with bovine serum albumin (RIA Grade, Fraction V, Sigma, USA), and then obtained results were described as μg of protein per mL of sample. Because the unit of protein contents is shown in weight per volume, assuming that the density (or specific gravity) of each preparation is 1.0.

4 Determination of bacterial endotoxin levels

The determination of bacterial endotoxin levels was performed according to the method described in Pharmacopoeias[14~16]. The endotoxin test was performed in triplicate on aliquots of test preparations. The test preparations were serially diluted to yield absorbance values falling within the working range of the standard curve. A 0.1 mL of the serially diluted test preparation or a series of endotoxin standard solution was separately placed in test tubes. A 0.1 mL of the main reagent (Toxicolor system LS-50M set : Main reagent which releases *p*-nitroaniline by reacting with bacterial endotoxin) was added to each tube. All tubes were mixed and incubated for 30 minutes at 37℃. Then, a 0.5 mL of the diazo-coupling reagent (Toxicolor system DIA set : Coloring reagent which produces azodye by reacting with *p*-nitroaniline) was added to each tube. Immediately after that, the absorbance was measured at 545 nm, the calibration curve was drawn and the concentration of the endotoxin determined in appropriate dilutions of the test preparations in triplicate. The resulting average value was as a result of endotoxin content for each test preparation.

5 Estimation of nucleic acids/DNA content

The UV spectrum between 190 and 400 nm was measured for all original test preparations in a spectrophotometer (UVIDEC-610, Japan Spectroscopic Co., Ltd.) using quartz cells. Care was taken to avoid air bubbles when placing the samples into the quartz cells. The spectra of several preparations showed a typical peak or shoulder indicating the presence of nucleic acid group at or around 260 nm. We, therefore, estimated the content of nucleic

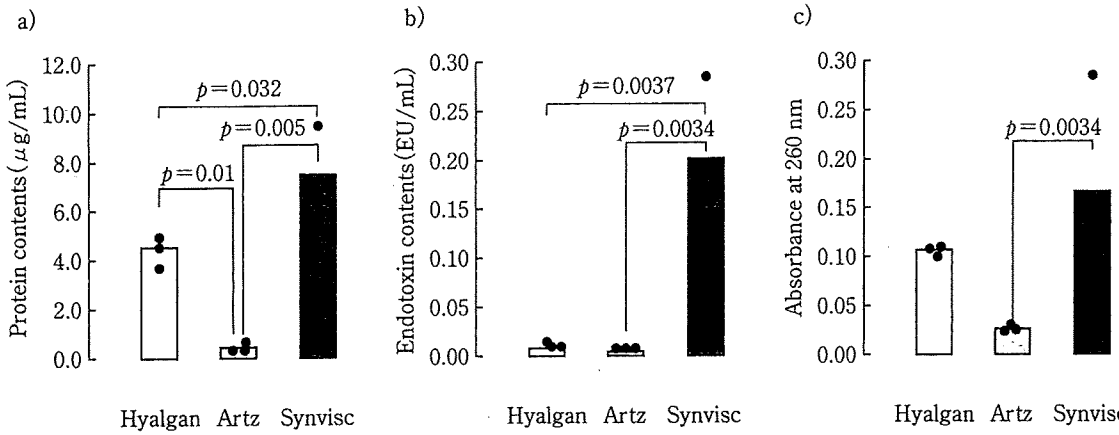

Fig. 1 Comparison of protein contents (a), bacterial endotoxin contents (b) and nucleic acids/DNA content estimated by absorbance at 260 nm (c) in HA preparations

Each bar indicates the mean of 3 lots and filled circles indicate raw data.

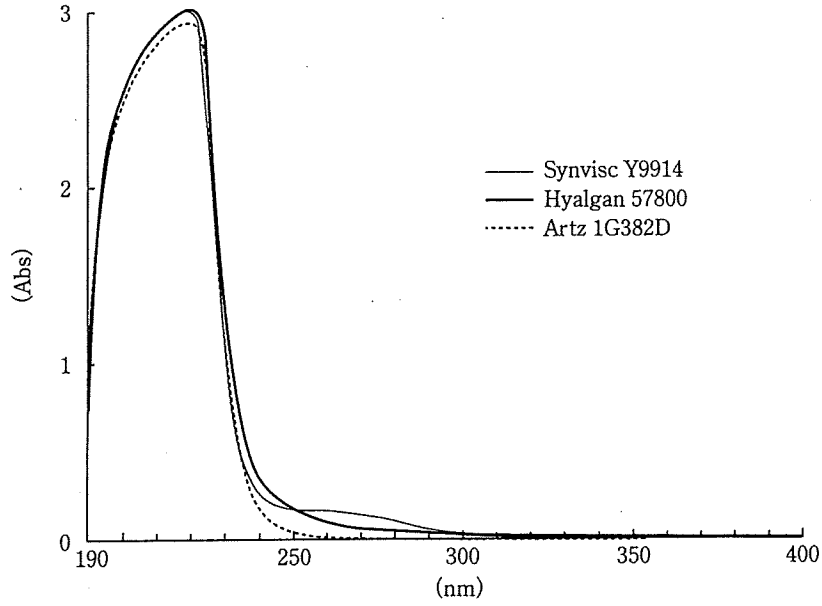

Fig. 2 Representative UV spectra of test preparations

acids/DNA by using the absorbance at 260 nm[17].

6 Statistical analysis

The statistical analyses are shown as the mean ± standard deviation (M ± SD) of three lots. Differences among each pair comparison by the Tukey method ; All tests controlled of the Type I error rate. p-values ≤ 0.05 was considered to indicate statistical significance.

Results

1 Protein content

The protein content of each test preparation is shown in **Fig. 1a**. Artz® had the lowest protein level (0.5 ± 0.1 μg/mL). Hyalgan® had a protein level (4.5 ± 0.6 μg/mL) that was about 9 times higher than that of Artz®

($p = 0.0100$). The highest protein level was observed in Synvisc®, whose level ($7.5 \pm 1.8\,\mu g/mL$) was 15 times higher than that of Artz® ($p = 0.0050$) and was over 1.5 times higher than that of Hyalgan® ($p = 0.0321$).

2 Bacterial endotoxin content

As shown in **Fig. 1b**, Artz® had the lowest endotoxin levels ($0.0045 \pm 0.0001\,EU/mL$), followed by Hyalgan® ($0.0074 \pm 0.0026\,EU/mL$). The highest endotoxin levels were observed in Synvisc®, whose level ($0.1991 \pm 0.0741\,EU/mL$) was over 40 times higher than that of Artz® ($p = 0.0034$) and was over 25 times higher than that of Hyalgan® ($p = 0.0037$).

3 UV spectra and nucleic acids/DNA content estimated by absorbance at 260 nm

Representative UV spectra of the three test preparations are shown in **Fig. 2**. On the spectra for Synvisc®, a typical peak indicating the presence of nucleic acids/DNA was found at or around 260 nm.

The absorbance at 260 nm of each test preparation was also measured. Among the three test preparations, Artz® showed the lowest absorbance. As shown in **Fig. 1c**, Hyalgan® and Synvisc® each had an average absorbance that was 4 and 6 times higher than that of Artz®, respectively (Synvisc® > Artz® : $p = 0.0153$).

Discussion

HA preparations have been roughly classified into two types : non-modified native HA products and chemical-modified (i.e., cross-linked) HA products. Hyalgan® and Artz which are used in this study are classified as non-modified HA products, and Synvisc® belongs to chemical-modified HA product to confer a high viscoelasticity to joint fluid and to remain in the joint cavity longer than the non-modified HA preparations[4]. In order to achieve this, Hylan®, the active substance in Synvisc®, is prepared by cross-linked HA using proteins from chicken combs[4,7].

As shown in **Fig. 1a**, the residual protein content of Synvisc® was found to be higher than that of the other test preparations. The high protein content of Synvisc® is inevitable, given its genesis. Compared with Hyalgan® and Artz®, Synvisc® had extremely high-level endotoxins and also high absorbance at 260 nm derived from nucleic acids/DNA (**Fig. 1b, 1c**). These results indicate that Synvisc® contains not only proteins but also endotoxins and nucleic acids/DNA at higher concentrations than the other test preparations.

It is well known that purified native HA itself has no immunogenecity[18], however several reports have shown that clinical complications such as SAIRs accompany with immunine response are related to cross-linked HA[10,11,19], and evidence for its immunogenecity has been accumulating by animal tests[20~23].

By recent discovering of Toll-like receptors (TLR) and analyzing of their functions, it has indicated that innate immunity (is not non-specific, but is specific enough to discriminate self from pathogens through evolutionarily conserved receptors,) plays a crucial role in the early host defense against invading pathogens, and activation of it is a prerequisite to the induction of acquired immunity[24]. LPS (bacterial endotoxins) is used commonly as an inflammatory substance and shown to work as an adjuvant at immunization[25~28], endotoxins and DNA have also been reported as inducing substances of acute arthritis[29,30]. Especially, it is reported that intra-articular injection of a small dose endotoxin at 0.125 ng/joint (equivalent to that of 1.25 EU/joint[31]) into healthy joint of horses causes synovitis[32]. As related to TLR recent study showed that endotoxin contamination in recombinant human heat shock protein 70 is responsible for induction of tumor necrosos factor α release by murine macrophages, endotoxin activies e.g. 0.3-1 EU/mL (equivalent to that of 0.1-0.3 ng/mL LPS) were sufficient to induce the cytokine production[33]. Endotoxins is potent substance that secretes various kinds of mediators[26,33], activates B cells, resulting in

marked production of polyclonal antibodies[25], and plays a role in some diseases including arthritis in which autoantibodies or self-specific T cells are involved[27, 28]. In OA joints threshold of inducing inflammatory reactions is supposed to be lower level than that of healthy joint. However the deviation of endotoxin level in cross-linked HA was quite high (e.g. as 0.28-0.57 EU/2 mL/human joint), which remains a small safety zone, therefore that may be related with inflammatory reactions and easier induction of acquired immunity of protein from chicken combs, even though HA per se has anti-inflammation effects[1, 34].

Some reports have argued about the etiologies of immunogenicity and granulomatous inflammation, and their possible factors ; chemical cross-linking, proteins and other components of rooster comb, purification process so on[35, 36], but lacking of analysis data of HA preparations. From our results and the above references, we suppose that cross-linked HA product such as Synvisc® would be possible to become a potent immunogen and inflammatory substance, especially in the case of endotoxin works in cooperation with others. At first endotoxin content of Hyalgan® was far superior to that of Synvisc®, relatively closer to that of Altz, however protein and DNA levels of Hyalgan® were not superior (**Fig. 1**). In the second place activation of TLR by endotoxin secreted mediators even though at very low concentration[33], resulting in induce of acquired immunity[24].

Since 1987 approximately 10 million injections of Hyalgan® and more than 100 million injections of Artz® have been given, and their post marketing surveillances have indicated that there are no differences of their frequencies, severities and sorts of symptoms among the cases of a single injection and over 20 time injections, or a single course treatment and repeated course treatment[8, 37, 38]. On the other hand, increased frequencies of acute reactions have reported in patients receiving more than one course treatment in the cross-linked HA[39]. Animal study of intra-articular injections of HA preparations indicated that after 3 weekly injections of cross-linked HA caused inflammation in about 50%, accompanied with high percentage eosiophils in synovial fluid and synovial membrane of slightly inflamed joints[35].

From the standpoint of safety profile for the treatment of OA, therefore, acute inflammatory reaction in patients receiving a particular preparation may be related with the differences in their possible impurities.

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Topical Reactions Against Intracutaneous Injection of Three Hyaluronan Products, Artz®, Synvisc® and Durolane® in Guinea Pigs

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ABSTRACT

The aim of the present study was to compare skin reactions against three hyaluronan (HA) products : Artz®, a purified natural HA obtained from chicken comb ; Synvisc®, a chemically cross-linked chicken comb HA ; and Durolane®, a biosynthetic HA with molecular cross-linking. These HA products were intracutaneously injected to the back of guinea pigs and the animals were observed for skin reactions for 28 days. Both Synvisc® and Durolane® resided longer in the tissue than Artz®. However, the cross-linking HA products caused erythema, and moreover, Synvisc® exhibited delayed skin reaction. In passive cutaneous anaphylaxis test only the sera from Synvisc®-treated animals showed a significant titer to Synvisc®, whereas sera from the animals treated with Durolane® or Artz® had no effects. Therefore it is possible that some of cross-linked HA products show antigenicity which might be due to contaminated antigenic and/or antigen-inducing substances, though such products have an advantage of longer residence time. In contrast natural molecule HA products appear far less inflammatogenic, while its residence time is somewhat shorter. (**Jpn Pharmacol Ther 2004 ; 32 : 663-77**)

KEY WORDS Hyaluronan, Cross-linking, Guinea pig, Skin reaction, Antigenicity

Introduction

Hyaluronan (HA), linear polysaccharide molecules consist of repeated disaccharide of O-β-D-glucuronosyl (1 $\rightarrow$ 3)-*N*-acetyl-β-D-glucosaminyl (1 $\rightarrow$ 4) is found ubiquitously in animal tissues, such as synovial fluid, umbilical cord and vitreous humor, and its elastoviscous property plays an important role in the maintenance of biologic homeostasis. Purified HA has been used for tissue engineering and ingredients in drugs and cosmetics. Elastoviscosity is indispensable to keep peculiar function of the joint, for pathologic conditions, such as chronic idiopathic osteoarthritis accompanied with decreased elastoviscosity in the joint[1, 2]. Thus, intra-articular injection of HA products is an epochal treatment which gives elastoviscosity to synovial fluid.

Purified non-inflammatory fraction of HA was first introduced by Balazs[3, 4] as a medical device for arthritic joint. The intra-articular injection of HA relieves pain, reduces cell adhesion and improves function in arthritic

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joints. In the 1980's hylans, HA derivatives with molecular cross-linking were engineered to give a longer intra-articular residence to HA[5,6]. The viscosupplementation with HA products has been reported to be effective and safe in clinical use regardless of molecular weight or existence of molecular cross-linking[7~9]. HA derivatives with molecular cross-linking are favorable for viscosupplementation and have been widely used, for the prolonged residence time of the products[10]. Recently, however, there is growing evidence that HA products with molecular cross-linking such as Synvisc® frequently reveal adverse effects as compared with purified intact HA products. That is, intra-articular injection of Synvisc® was found to be associated with significant acute local inflammation that persisted for 3 weeks[11], gout[12], recurrent calcium pyrophosphate dihydrate arthritis or pseudogout[13~15]. Moreover, it has been reported that intra-articular injection of Synvisc® caused inflammation of the synovial membrane and an increase in white blood cell count in the synovial fluid of rabbits[16], and Synvisc® was confirmed to have immunogenicity[17]. On these knowledge, we aimed in the present study, to compare skin reactions of guinea pigs against three HA products : Artz®, a purified natural HA obtained from chicken comb ; Synvisc®, a chemically cross-linked chicken comb HA ; and Durolane®, a biosynthetic, non-animal stabilized HA with molecular cross-linking[18]. We also attempted to examine whether or not these products reveal immunogenicity. For this purpose, we injected intracutaneously these products to the back of guinea pigs and observed for skin reactions for 28 days. In addition, passive cutaneous anaphylaxis test was also done using sera obtained from these animals.

Materials and Methods

1 Chemicals

Artz® (Seikagaku, Japan), Synvisc® (Biomatrix, UK) and Durolane® (Q-Med, UK), each of these contains 10 mg sodium hyaluronate from chicken comb, 8 mg chemically cross-linked sodium hyaluronate from chicken comb and 20 mg chemically cross-linked biosynthetic sodium hyaluronate per 1 mL of buffered saline, respectively. These HA products were kept in a refrigerator at 2～7℃ until use. All other chemicals used were of commercially available.

2 Animals

Sixty-one male guinea pigs of Hartley strain (Japan Laboratory Animal Inc.), 6-week old, weighing 346 to 496 g were used. They were individually placed in metal hanging cages with metal mesh bottom (260W × 380D × 200H mm). The temperature and relative humidity surveyed in the rearing room were 22.5～23.5℃ and 50.5～74.5%, respectively. The room was ventilated approximately 15 times/hour and lighting cycle was 12 hours (on, from 7.00 to 19.00 ; off, from 19.00 to 7.00). The animals were allowed to receive a pellet chow (CG-7, Oriental Yeast, Japan) and tap water freely.

The animals were used following the instructions of the Committee for Ethical Usage of Experimental Animals in Hatano Research Institute.

3 Test for skin reactions

The experiment was carried out being based on ISO standard 10993-10, Biological evaluation of medical device-Part 10 : Tests for irritation and sensitization. The animals were divided into 3 groups of 15 animals by stratified random sampling on the basis of body weight.

Forty-five animals, whose furs on the back were clipped, were anesthetized by intraperitoneal 32.4 mg/kg sodium pentobarbital. Artz®, Synvisc® or Durolane® was injected intracutaneously at 4 points, 2 points on each side

Table 1 Scoring system for skin reaction

Reaction	Score
1) Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4
2) Oedema formation	
No edema	0
Very slight edema (barely perceptible)	1
Well-defined edema (edges of area well-defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond exposure area)	4
Total possible score for irritation	8

of the back at the cranial portion making parallel line with the spine, and physiological saline (Otsuka, Japan) was also injected at 4 points of the caudal portion. The volume of the solutions injected was 0.2 mL for each point.

The animals were examined daily for general condition and skin reactions at the points injected for 28 days. Erythema and incrustation, and edema formation were scored according to Draize's standards for scoring skin responses (**Table 1**). Other findings of the skin were recorded, if any.

The animals were weighed on the next day of, and the 3rd, 7th, 14th and 28th day from the injection. Three animals of each group were selected following numerical orders, anesthetized with sodium pentobarbital and killed by cervical dislocation at each of the above-mentioned days. The skin of the back was removed and immersed in the fixate, 0.1M phosphate buffered 10% formalin solution. Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The specimens of areas injected with HA products were examined under a microscope. A part of the specimens were stained with Alcian blue, and compared with those treated with hyaluronidase prior to Alcian blue staining to identify resident polysaccharide injected.

4 Passive cutaneous anaphylaxis (PCA) test

Serum was obtained from each animal in the above-mentioned experiment when the animal was killed. Twelve guinea pigs were clipped furs on the back, and 6 to 7 different sera from the individual animals and saline were intracutaneously injected at a volume of 0.1 mL for each, making bilateral parallel lines with the spine. Approximately 20 hours after the injection, 0.2 mL of 1% Evans blue saline solution was injected intravenously with 1 mL of saline containing 1 mg/mL Artz®, Durolane® or Synvisc®. Thirty minutes after the injection, the animals were killed by euthanasia. The skin of the back was removed, placed face down and examined for blue foci by leakage of the dye from subcutaneous micro vessels. When foci, 5 mm or larger in diameter were found, sera injected were evaluated to cause positive reaction. The sera from 3 animals killed at the 1st day, from 3 animals killed at the 3rd day and from 2 animals killed at the 7th day were injected to one animal. The serum from another animal killed at 7th day, the sera from 3 animals killed at 14th day and from 3 animals killed at the 28th day were injected to another animal. Two animals were used for each treatment for confirming reproducibility of the reaction.

Titer unit of antibody was determined when PCA-positive response was found for sera from the Synvisc®-treated animals. Namely, another PCA test was carried out in 2 animals using diluted sera from the Synvisc®-treat-