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Toxicity Study of *Streptococcus pneumoniae* Vaccine Administrated Subcutaneously in Rats

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This study was performed to evaluate the toxicity of polysaccharide-based *Streptococcus pneumoniae* vaccine in Specific Pathogen Free (SPF), Sprague-Dawley (SD) rats. *S. pneumoniae* vaccine was administrated subcutaneously each dose level of high (560 μg/rat), medium (280 μg/rat) and low (140 μg/rat) on days 0, 14, 28. The rats were observed for 2 weeks or 4 weeks after the final injection. During this test, there were no significant dose-dependent changes in body weight, water and food consumption. In urinalysis and serum chemistry, dose-related changes were not detected. In hematology, the percent of neutrophils and lymphocytes in white blood cells were changed significantly. According to the measurement of organ weight, only spleen weight was significantly increased in all groups of administration compared to the control group. In the histopathological examination, an antigen-deposit, vacuolated macrophages, infiltrated inflammatory cells and a formation of granulation tissue were observed at the site of an administration. These results are considered as an outcome by immune responses through a vaccination. Consequently, the results of this study demonstrated that *S. pneumoniae* vaccine has no toxicity when it was administrated subcutaneously three times in 2-week interval at a high dose of 560 μg/rat.

Key words: *Streptococcus pneumoniae*, Polysaccharide, Vaccine

INTRODUCTION

Streptococcus pneumoniae is an ubiquitous human pathogen and causes diseases ranging from localized infections to life threatening invasive diseases, such as acute otitis media, pneumonia, meningitis, and bacteremia (Ring *et al.*, 1998; Hausdorff *et al.*, 2005). And *S. pneumoniae* has capsular polysaccharides (PSs) that have been found to be important in infective diseases. The PSs are serotype-specific and main antigenic determinants of *S. pneumoniae* (Black *et al.*, 2000). However, PSs are T cell independent antigens. They stimulate mainly an immunoglobulin M (IgM) antibody with an weak memory.

The currently available pneumococcal vaccines are 23-valent pneumococcal polysaccharide vaccine, 7-valent and 13-valent pneumococcal conjugate vaccine. Pneumococcal conjugate vaccine, which has been used in infants and children worldwide, increases immune responses through a carrier protein conjugated to PSs.

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In particular, 7-valent pneumococcal vaccine (Prevenar, Wyeth) was the most imported complete drug in the 2006 and 2007. In 2007, it was imported 45,433 thousand U.S. dollars and this showed about 52% of the growth rate compared to 2006 (Food and Drug Statistics Yearbook, 2008). Considering the imported 23-valent pneumococcal polysaccharide vaccine, the domestic demand would be much greater. However, the conjugate pneumococcal vaccine produces a higher level specific antibody against a carrier protein than that against PSs and it is useless in attacking pneumococcal strains (Meng *et al.*, 2009). Moreover, we require to have an autonomous management of a vaccine supply and demand in a domestic market. To solve these problems, it is demanded to develope the improving our own vaccine.

In this study, a repeated-dose administration test was performed as a part of a safety assessment of pneumococcal vaccine, which does not use a carrier protein but apply a new adjuvant known as a liposome. This vaccine was developed by Jeollanamdo BioPharmaceutical Research Center (JBRC) and injected three times to rats subcutaneously at every 2-week for 6 weeks. Clinical symptoms were observed during the test period. And histopathological tests including an autopsy and pathological test were performed after completion of tests to evaluate the safety of the test material.

Table 1. Composition of test group and administration condition

Group	Test material	Sex	Number		Dose μg/rat	Volume ml//rat	Day
			Test	Recovery			
Control	PBS	male	10	5	0	1.72	0, 14, 28
		female	10	5			
Low dose		male	10		140	1.72	0, 14, 28
		female	10				
Medium dose		male	10		280	1.72	0, 14, 28
		female	10				
High dose		male	10	5	560	1.72	0, 14, 28
		female	10	5			

MATERIALS AND METHODS

Test material. Test material, which is a vaccine against *Streptococcus pneumoniae*, was prepared at the laboratory scale in JBRC. PSs as an antigen were prepared from the cultivation of *S. pneumoniae* by alcohol precipitation using a modified method (Daoust *et al.*, 1981). And *S. pneumoniae* serotypes 3, 4, 6A, 6B, 7F, 9V, 14, 15, 18C, 19A, 19F and 23F were included in this test material. Then these PSs were entrapped in liposomes, which composed of phosphatidylcholine, cholesterol and stearylamine. It is form of slightly turbid liquid. And it was stored in the refrigerator until used. The concentration of test material was adjusted using Phosphate buffered saline (PBS) depending on the dose of each group. PBS buffer was used in the control group.

Animals and environmental conditions. Six-week old male and female, specific pathogen-free (SPF) Sprague-Dawley (SD) rats were purchased from Orient Bio Inc. Healthy 50 rats of each male and female were used in this study after acclimation for 5 days observing the clinical symptoms. During the acclimation and experimental periods, the rats were housed in an animal room with controlled temperature (22 ± 3)°C, relative humidity (50 ± 20)%, ventilation rate (10~15) times/h, illumination (150~300) Lux and a 12 hour light/dark cycle. Irradiated (2.0 Mrad) diet and UV irradiated water were fed to rats.

Test group and the test methods. Based on the amount of PSs contained in 23-valent pneumococcal polysaccharide vaccine and 7-valent pneumococcal conjugate vaccine, 140 μg (0.5X), 280 μg (1X) and 560 μg (2X) were determined as a low dose, a medium dose and a high dose, respectively in this study. An efficacy test was also considered when the amount of an injection was determined. In addition, recovery groups were composed to evaluate the persistence and reversibility of toxic effects of the test material in the high dose group and the control group. Recovery groups were observed for 2 weeks after the end of the test 6-week, then performed the autopsy. Test material was injected three times subcutaneously with 2-week intervals. And the injection volume was adjusted equally to 1.72 m*l*/rat with PBS buffer, as the volume of a high dose group, in all groups. Rats in test groups were sacrificed 6-week after (Table 1).

Clinical signs. All animals were observed clinical signs, toxicity effect everyday. Behavior, breath, alopecia, urination, defecation, lacrimation, ptosis, edema, phonation, ataxia, sensory function, muscular tension and trauma are included in the clinical signs.

Body weight, food and water consumption. Body weight was determined when receipt, grouping, before injection and every week. We calculated the consumption average (g/rat/day) through measuring the rest of the food and water on the day after feeding.

Opthalmological examination. Appearance of eyes was observed with naked eyes on the last week of experiment. The eyes of all control and high dose animals were examined by ophtalmoscope (Genesis, Kowa, Japan). Opthalmological examination was observed by Ocutropine (Lot. No. 018648, Samil pharm CO., LTD, Korea).

Urinalysis. On the last week, urinalysis was conducted with urine to determine glucose, bilirubin, ketone, specific gravity, blood, pH, protein, urobilinogen, nitrite and leukocyte by using Multistix 10SG (Bayer, U.S.A.) and urine analyzer (Clinitek 500, U.S.A.). The color tone and transparency were measured with fresh urine collected for 3 hours. During the collection, the rats were housed in metabolism cages which allowed for separate collection of urine and feces.

Hematological analysis. The animals were fasted overnight and blood was collected from abdominal artery under anesthesia with isoflurane. Three milliliter of blood was

transfer into CBC bottle (EDTA 3K, BD, USA) and analyzed using autohematoanalyzer (ADVIA120E, Seimens, USA). On the other hand, blood was transfer into vacutainer (Sodium citrate 3.2%, BD) and centrifuged at 3000 rpm for 10 min. The plasma was isolated and used to determine aggregation time using coagulometer (ACL 7000, WERFEN MEDICAL IL, USA). Following items were analyzed: WBC (white blood cell count), RBC (red blood cell count), Hb (hemoglobin concentration), HCT (hematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), RETI (reticulocyte), PT (prothrombin time), APTT (activated partial thromboplastin time) PLT (platelet counts), NE% (percent of neutrophils), LY% (percent of lymphocytes), MO% (percent of monocytes), EO% (percent of eosinophils) and BA% (percent of basophils).

Blood chemistry. Serum was isolated and used for biochemical analysis using automatic serum analyzer (Hitachi 7060, Japan) and electolyte analyzer (EasyLyte PLUS Na/K/Cl Analyzer, WERFEN MEDICAL IL, USA).

Necropsy findings. After blood was collected under deep anesthesia with isoflurane, the animals were sacrificed. Gross observation was performed against external surface, all orifices, cranial cavity and all organs in thoracic and abdominal.

Organ weight. After necropsy, the absolute and relative (organ-to-body weight ratios) weight of following organs was measured in all survivors when they were sacrificed: liver, kidney, spleen, adrenal gland, testis, ovary, brain, pituitary, lung, heart, thymus, prostate gland, and uterus.

Histopathology of injection site (local resistance). Isolated areas of injection site for all animals were fixed in 10% neutral buffered formalin solution for pathological examination.

Histopathology. The following tissues were obtained from all animals : liver, kidney, adrenal gland, heart, lung, brain, pituitary gland, spleen, seminal vesicle, testis, ovary, epididymis, uterus, prostate gland, vagina, tongue, trachea, esophagus, thymus, thyroid gland, stomach, duodenum, urinary bladder, small/large intestine, eyeball, submandibular gland, pancreas, and mesenteric lymph node. Eyeball and testis were preserved in Davidson's fixative and Bouin's fixative, respectively. Other tissues were fixed with 10% neutral buffered formalin solution. The tissues from only high dose group and control group were processed to take histopathology test. But histopathology of injection site and spleen were examined in all test groups.

Statistical analysis. The body weight, hematological data, biochemistry data and organ weight were analyzed for homogeneity of dispersion using Levene's test. One way ANOVA analysis was performed for data recognized the significance of dispersion. Homogeneity of dispersion and significance between test groups is recognized, the Scheffe's test was conducted. Heterogeneous of dispersion and significance between test groups is recognized, Dunnett's T3 was conducted. Recovery groups were analyzed using Students T test. All analysis were performed using SPSS program.

RESULTS

General symptoms and mortality. At the administrated site of the test material, the symptom of skin swelling was observed from the 1st injection and maintained until an autopsy. In some parts of the test group, wounds of the administrated site was observed on day 11 after the 1st injection. Some wounds were recovered and some were continued until autopsy. The wound of injection site was observed at a higher frequency as a dose-dependent manner. During the test period, dead animals from all experimental groups were not observed.

Weight. During the trial, animals showed weight changes. Weight of male rats in low, medium and high dose groups decreased significantly compared to the control group ($p < 0.05$, $p < 0.05$, $p < 0.01$) after the 1st injection. However, all test groups showed no significant difference after the 2nd injection. In the female rats's body weight, there was no significant difference compared to control group (Fig. 1, 2).

Consumption of food and water. Food consumption of male rats in the high dose group was increased signifi-

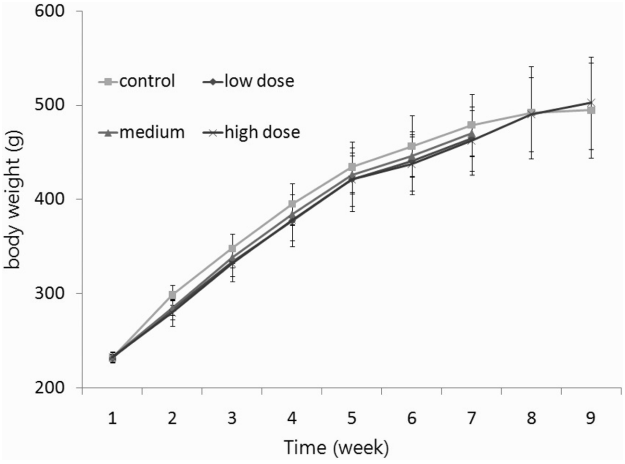

Fig. 1. Body weight change in male rats administrated with *S. pneumoniae* vaccine subcutaneously at a dose level of high (560 μg/rat), medium (280 μg/rat) and low (140 μg/rat). There were no significant differences between control and other treatment groups.

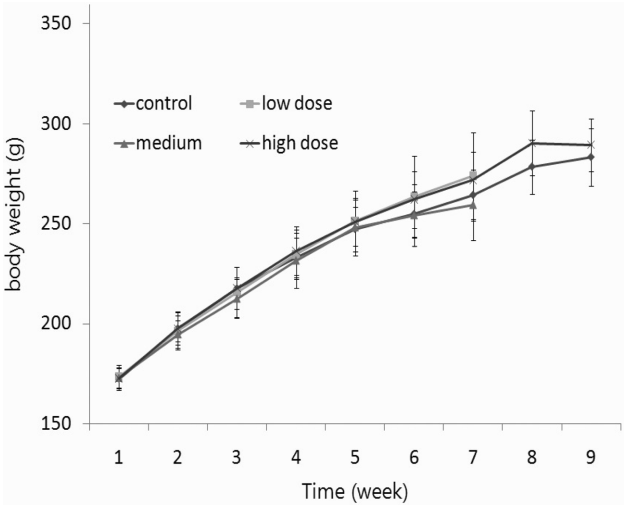

Fig. 2. Body weight change in female rats administrated with *S. pneumoniae* vaccine subcutaneously at a dose level of high (560 μg/rat), medium (280 μg/rat) and low (140 μg/rat). There were no significant differences between control and other treatment groups.

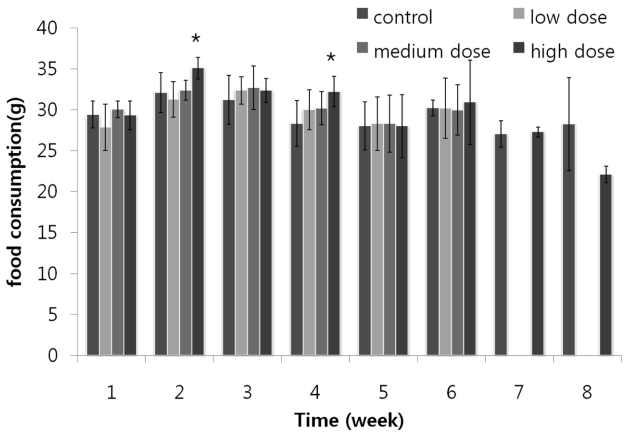

Fig. 3. Food uptake in male rats administrated with *S. pneumoniae* vaccine subcutaneously. * P < 0.05.

cantly at 2 and 4-week of test period compared to the control group ($p < 0.05$). Food consumption of female rats in the high dose group was increased significantly at 4-week of test period and 1-week of recovery period compared to the control group ($p < 0.05$, $p < 0.01$) (Fig. 3, 4). In case of water intake, male rats in the high dose group showed significant increase at 2-week of test period compared to the control group ($p < 0.01$). And female rats in the medium dose group showed significant increase at 1 and 2-week of test period compared to the control group ($p < 0.05$, $p < 0.01$) (Fig. 5, 6).

Although consumption of food and water in some test groups showed significant results compared to the control group, it was not a dose- dependent manner and was temporary. Thus, it is not considered as a toxicological change caused by the test material.

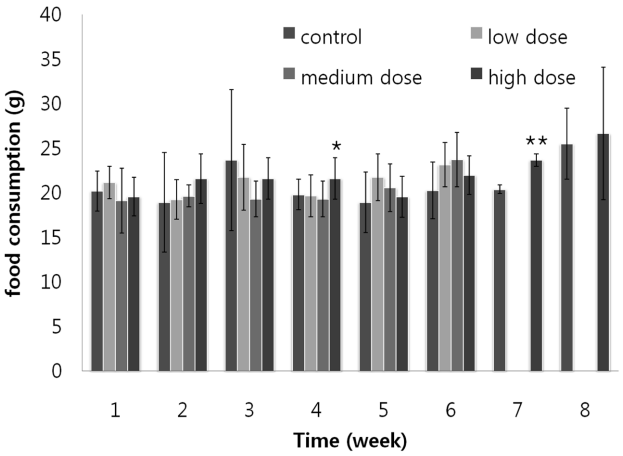

Fig. 4. Food uptake in female rats administrated with *S. pneumoniae* vaccine subcutaneously. * P < 0.05, ** P < 0.01.

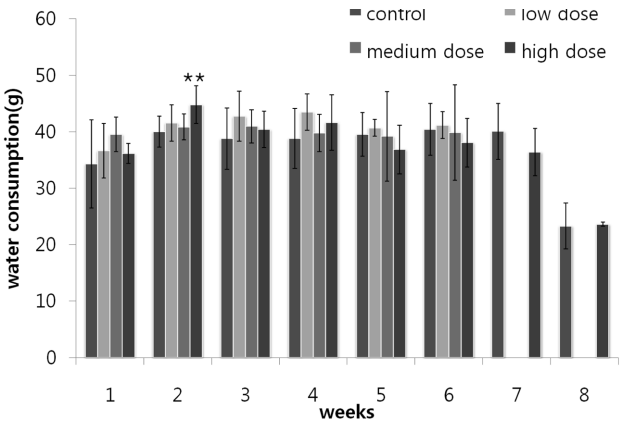

Fig. 5. Water consumption in male rats administrated with *S. pneumoniae* vaccine subcutaneously. ** P < 0.01.

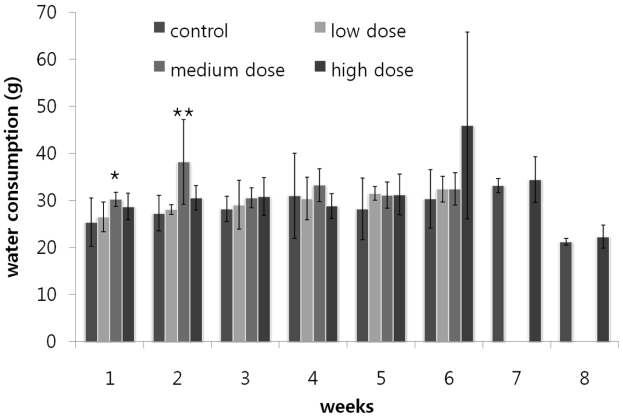

Fig. 6. Water consumption in female rats administrated with *S. pneumoniae* vaccine subcutaneously. * P < 0.05, ** P < 0.01.

Table 2. Group mean hematology values

Sex	Dose (μg)	No. of rat	WBC	WBC differential counting (%)					RBC	Hb	HCT	MCV	MCH	MCHC	RETI	PLT	PT	APTT
			(K/μg)	NE	LY	MO	EO	BA	(M/μg)	(g/dl)	(%)	(fL)	(pg)	(g/dl)	(%)	(K/μg)	(sec)	(sec)
male	0	10	5.52 ± 1.65	16.5 ± 4.9	79.0 ± 5.6	2.4 ± 1.2	1.4 ± 0.5	0.3 ± 0.2	7.73 ± 0.54	14.3 ± 0.7	42.6 ± 2.2	55.2 ± 2.1	18.6 ± 0.6	33.6 ± 0.5	2.82 ± 1.15	1152 ± 86	15.7 ± 1.8	21.5 ± 1.0
	140	10	8.09 ± 1.91	26.8* ± 7.0	68.9* ± 7.1	2.5 ± 1.1	1.2 ± 0.6	0.2 ± 0.1	8.07 ± 0.18	14.3 ± 0.4	42.8 ± 1.1	53.0 ± 1.4	17.7* ± 0.5	33.4 ± 0.5	3.56 ± 1.82	1210 ± 130	15.8 ± 2.0	21.1 ± 1.0
	280	10	9.49** ± 2.60	30.4** ± 7.5	65.9** ± 7.5	2.3 ± 0.6	0.8 ± 0.3	0.3 ± 0.1	7.51 ± 0.39	13.6 ± 0.6	40.7 ± 1.5	54.3 ± 1.5	18.1 ± 0.6	33.4 ± 0.4	3.43 ± 0.79	1204 ± 126	16.9 ± 2.0	21.8 ± 2.2
	560	10	10.77** ± 2.58	32.2** ± 7.5	63.9** ± 7.8	2.2 ± 0.8	0.9 ± 0.3	0.3 ± 0.1	7.98 ± 0.52	13.8 ± 1.0	42.0 ± 3.1	52.6* ± 1.6	17.3** ± 0.5	33.0* ± 0.3	3.39 ± 0.74	1287 ± 139	16.2 ± 1.6	20.8 ± 1.7
	0 (recovery)	5	7.03 ± 1.32	16.7 ± 6.9	79.0 ± 7.8	2.7 ± 1.1	1.0 ± 0.2	0.2 ± 0.1	7.81 ± 0.33	13.8 ± 0.2	40.7 ± 0.3	52.2 ± 2.3	17.7 ± 0.6	33.9 ± 0.3	2.27 ± 0.22	925 ± 162	15.5 ± 1.9	21.0 ± 1.3
	560 (recovery)	5	5.73 ± 1.01	27.7 ± 9.9	67.0 ± 9.2	2.4 ± 0.3	2.5 ± 1.5	0.1 ± 0.1	8.02 ± 0.17	14.0 ± 0.5	41.7 ± 1.7	52.0 ± 2.6	17.5 ± 0.7	33.7 ± 0.4	2.00 ± 0.28	995 ± 99	14.4 ± 1.5	20.1 ± 2.3
female	0	10	4.64 ± 1.22	11.3 ± 3.4	84.0 ± 4.3	2.4 ± 1.1	1.5 ± 0.5	0.2 ± 0.1	7.25 ± 0.62	13.4 ± 0.8	39.2 ± 2.1	54.2 ± 2.1	18.5 ± 0.7	34.2 ± 0.5	3.07 ± 1.63	1203 ± 118	13.3 ± 0.3	16.3 ± 1.1
	140	10	5.92 ± 1.41	25.1* ± 10.4	70.7** ± 10.0	2.2 ± 0.7	1.2 ± 0.4	0.3 ± 0.2	7.35 ± 0.41	13.6 ± 0.7	40.5 ± 2.1	55.1 ± 2.3	18.5 ± 0.7	33.6* ± 0.3	3.83 ± 1.24	1248 ± 85	13.8 ± 0.9	16.0 ± 0.8
	280	10	6.39 ± 2.28	18.9** ± 5.0	76.7 ± 5.8	2.3 ± 1.2	1.2 ± 0.7	0.2 ± 0.1	7.50 ± 0.50	13.8 ± 0.7	40.9 ± 1.6	54.6 ± 1.7	18.4 ± 0.4	33.6 ± 0.5	3.21 ± 1.17	1295 ± 152	13.5 ± 1.0	16.0 ± 1.7
	560	10	7.13* ± 1.90	23.9** ± 6.9	72.1** ± 7.1	2.5 ± 0.6	0.9 ± 0.2	0.2 ± 0.1	7.02 ± 0.32	13.2 ± 0.6	39.2 ± 1.6	56.0 ± 1.8	18.8 ± 0.6	33.6 ± 0.5	4.24 ± 1.14	1425** ± 139	13.0 ± 0.7	15.3 ± 1.2
	0 (recovery)	5	2.93 ± 0.55	21.9 ± 7.8	73.7 ± 8.4	2.0 ± 0.8	1.9 ± 0.4	0.2 ± 0.1	7.10 ± 0.20	13.4 ± 0.5	38.7 ± 1.3	54.5 ± 1.8	18.9 ± 0.4	34.7 ± 0.7	2.54 ± 0.67	1071 ± 112	14.1 ± 0.6	16.8 ± 0.5
	560 (recovery)	5	4.07 ± 2.08	23.6 ± 7.4	70.8 ± 7.6	3.1 ± 0.6	1.9 ± 0.5	0.1 ± 0.1	7.48 ± 0.26	13.7 ± 0.7	39.9 ± 1.9	53.4 ± 1.1	18.4 ± 0.5	34.4 ± 0.5	1.36 ± 0.34	1010 ± 41	13.7 ± 1.0	16.9 ± 1.1

Significant differences as compared with control: * $P < 0.05$, ** $P < 0.01$.

Table 3. Group mean blood chemical values

Sex	Dose (ug)	No. of rat	TP (g/d*l*)	ALB (g/d*l*)	A/G (g/d*l*)	T-BIL (g/d*l*)	ALP (U/*l*)	AST (U/*l*)	ALT (U/*l*)	CREA (mg/d*l*)	BUN (mg/d*l*)	CHOL (mg/d*l*)	TG (mg/d*l*)	GLU (mg/d*l*)	CA (mg/d*l*)	IP (mg/d*l*)	CK (IU/*l*)	Na mmol/*l*	K (mg/d*l*)	Cl (mg/d*l*)
male	0	10	5.8 ± 0.2	2.5 ± 0.1	0.8 ± 0.1	0.04 ± 0.01	473 ± 96	102 ± 9	31 ± 4	0.4 ± 0.0	11.5 ± 1.3	63 ± 14	62 ± 22	164 ± 24	10.1 ± 0.2	7.8 ± 0.6	408 ± 143	146.3 ± 1.3	4.51 ± 0.16	103.0 ± 1.7
	140	10	6.0 ± 0.3	2.5 ± 0.1	0.7 ± 0.0	0.06 ± 0.02	445 ± 82	106 ± 16	32 ± 5	0.4 ± 0.1	13.0 ± 0.8	67 ± 8	69 ± 29	155 ± 13	10.1 ± 0.3	8.2 ± 0.9	428 ± 132	146.1 ± 1.1	4.66 ± 0.27	103.0 ± 1.6
	280	10	5.8 ± 0.3	2.4* ± 0.1	0.7 ± 0.1	0.05 ± 0.01	451 ± 76	116 ± 25	33 ± 4	0.5 ± 0.1	13.1* ± 1.2	59 ± 14	63 ± 27	158 ± 20	10.0 ± 0.4	8.2 ± 0.7	473 ± 233	145.6 ± 1.1	4.73 ± 0.18	103.4 ± 1.5
	560	10	5.8 ± 0.2	2.4* ± 0.1	0.7 ± 0.1	0.05 ± 0.01	404 ± 92	114 ± 24	33 ± 5	0.4 ± 0.1	12.9 ± 1.3	57 ± 4	45 ± 12	155 ± 20	9.9 ± 0.3	8.1 ± 0.6	477 ± 276	145.8 ± 1.0	4.76 ± 0.28	103.0 ± 1.1
	0 (recovery)	5	5.6 ± 0.4	2.4 ± 0.1	0.7 ± 0.1	0.06 ± 0.01	356 ± 45	121 ± 13	35 ± 6	0.5 ± 0.1	12.8 ± 2.1	61 ± 12	61 ± 23	186 ± 10	9.6 ± 0.4	6.6 ± 0.7	513 ± 144	145.3 ± 0.8	4.41 ± 0.19	102.1 ± 1.6
	560 (recovery)	5	5.7 ± 0.3	2.4 ± 0.1	0.7 ± 0.0	0.05 ± 0.01	393 ± 37	114 ± 19	36 ± 6	0.5 ± 0.1	13.7 ± 2.0	71 ± 8	50 ± 30	181 ± 21	9.6 ± 0.3	6.7 ± 0.2	421 ± 142	146.2 ± 1.7	4.41 ± 0.17	103.0 ± 1.2
female	0	10	6.6 ± 0.2	2.9 ± 0.2	0.8 ± 0.1	0.07 ± 0.02	286 ± 62	99 ± 11	26 ± 4	0.5 ± 0.1	15.2 ± 3.7	73 ± 8	15 ± 5	151 ± 20	10.4 ± 0.3	6.9 ± 0.5	329 ± 77	145.3 ± 1.7	4.43 ± 0.27	103.2 ± 1.8
	140	10	6.4 ± 0.3	2.8 ± 0.2	0.8 ± 0.1	0.07 ± 0.02	320 ± 75	99 ± 12	27 ± 4	0.5 ± 0.0	14.4 ± 2.5	75 ± 17	13 ± 5	157 ± 26	10.3 ± 0.3	7.1 ± 0.6	334 ± 134	145.0 ± 1.8	4.41 ± 0.26	103.5 ± 2.5
	280	10	6.4 ± 0.3	2.8 ± 0.2	0.8 ± 0.1	0.06 ± 0.02	292 ± 88	97 ± 19	23 ± 3	0.5 ± 0.0	14.9 ± 2.1	73 ± 21	14 ± 9	148 ± 16	10.2 ± 0.3	7.0 ± 0.6	320 ± 171	145.2 ± 1.0	4.39 ± 0.24	103.6 ± 1.5
	560	10	6.5 ± 0.2	2.7* ± 0.1	0.7* ± 0.1	0.06 ± 0.02	286 ± 28	105 ± 15	24 ± 4	0.5 ± 0.0	14.5 ± 2.1	73 ± 14	13 ± 5	147 ± 12	10.2 ± 0.2	7.3 ± 0.3	388 ± 128	145.3 ± 1.2	4.48 ± 0.23	103.7 ± 1.0
	0 (recovery)	5	6.5 ± 0.3	2.9 ± 0.1	0.9 ± 0.1	0.07 ± 0.02	233 ± 55	109 ± 16	30 ± 4	0.6 ± 0.1	16.7 ± 1.7	73 ± 12	30 ± 24	157 ± 13	10.0 ± 0.2	12.8 ± 17.7	465 ± 164	145.3 ± 1.5	4.10 ± 0.21	103.8 ± 1.8
	560 (recovery)	5	6.5 ± 0.4	2.8 ± 0.2	0.8 ± .00	0.07 ± 0.01	252 ± 37	97 ± 19	30 ± 6	0.6 ± 0.1	16.5 ± 2.5	75 ± 12	20 ± 3	160 ± 15	10.1 ± 0.2	5.0 ± 0.4	292 ± 164	146.0 ± 1.0	4.06 ± 0.27	103.9 ± 0.7

Significant differences as compared with control: * $P < 0.05$.

Urinalysis, ophthalmic test, hematological, blood biochemistry. There were no specific symptoms in urinalysis, ophthalmic test. In the hematological test, the percent of neutrophils and lymphocytes in the white blood cells was changed significantly (Table 2). The data of blood biochemistry were explained in Table 3. However, it was not a dose-dependent manner and a gender-correlation. Thus, it is not considered as a toxicological change caused by the test material.

The autopsy and organ weight measurements. The autopsy showed that there were no remarkable abnormalities of major organs except the skin swelling at an injection site.

Absolute spleen weight of male rats in low and medium dose group increased significantly compared to the control group ($p < 0.05$) (Fig. 7). And also relative spleen weight of male rats increased significantly in the low, medium and high dose group ($p < 0.01$, $p < 0.01$, $p < 0.05$).

Absolute spleen weight of female rats in the low, medium and high dose group increased significantly compared to the control group ($p < 0.05$) (Fig. 8). And also relative spleen weight of female rats increased significantly in medium and high dose group ($p < 0.01$).

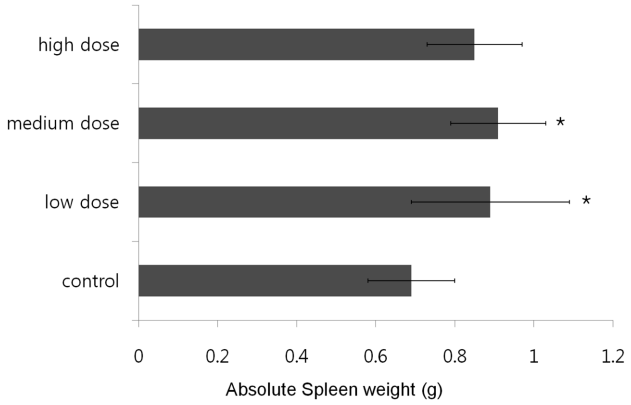

Fig. 7. Group mean absolute spleen weight in male rats administrated with *S. pneumoniae* vaccine subcutaneously. * $P < 0.05$.

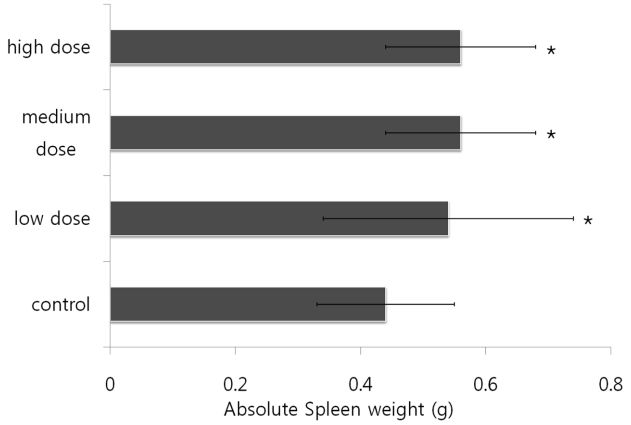

Fig. 8. Group mean absolute spleen weight in female rats administrated with *S. pneumoniae* vaccine subcutaneously. * $P < 0.05$.

Histopathological test. Overall, Lesions of injection sites were moderate in the 2nd and 3rd injection compared to the 1st injection. In case of the 1st injection, the granulation tissues and vacuolated macrophages around the injection site were distributed widely. In some cases, mild infiltration of inflammatory cells of various types was showed in the inside of granulation tissue. The 2nd and 3rd injection site showed similar lesions, but the extent was moderate.

In the same test group, a degree of lesions had a tendency to be stronger at closer to the autopsy and a dose-dependent manner. Except the lesion around the injection site, the pathologic lesions associated with the test material were not observed in major organs.

DISCUSSION

S. pneumoniae is a major cause of acute otitis media (AOM), pneumonia and bloodstream infections (bacteremia). The highest incidence of pneumococcal diseases occurs in the first few years of life and the patients who are under low immunity to infection. Current pneumococcal vaccines for infants are 7-valent and 13-valent Prevenar (Wyeth) which are chemically conjugated to carrier proteins.

These conjugate vaccines induce an unintended antibody against carrier proteins. Moreover, current studies reported that serotypes eradicated by the conjugate vaccine are being replaced by non-vaccine pneumococcal serotypes (Hausdorff *et al.*, 2005; Pletz *et al.*, 2008). In this study, the test material was prepared with liposome technology applied to polysaccharide of *S. pneumoniae* to increase ability of immune responses in infants (Fenske *et al.*, 2008; Rao *et al.*, 2000).

Test material was subcutaneously injected three times in 2-week intervals with 140 μg (0.5X), 280 μg (1X) or 560 μg (2X) /SD rats for investigation of toxicity in whole body and determination of non-toxic dose. The rats were investigated for 2 weeks after final injection to check toxicity. The recovery groups were observed additionally for 2 weeks to check recovery. A swelling was observed in the injection site. And the incidence and degree were dose-dependent manners. This swelling at the injection site is considered as the consequence of the injection volume. PSs 280 μg per 12-valent liposomal polysaccharide vaccine is determined as a clinical dose based on the current available pneumococcal vaccine. The 23-valent pneumococcal polysaccharide vaccine and 7-valent pneumococcal conjugate vaccine have 25 μg and 2 μg of PSs, respectively. And also, administration volume was determined considering the manufacturing process with JBRC. An injection volume was

adjusted to the same as that of the high dose group not to make any differences caused by the inequality of the injection volume. The volume (1.72 m*l*) would not be common to rats, but the guideline for preclinical test of biopharmaceutics is stating that the highest dose for human in clinical test can be applied to animal without conversion by body weight or body-surface area (Guideline on Nonclinical Evaluation of Biopharmaceuticals, 2008).

Consumption of food and water were changed temporally and it was a dose-independent manner even though some group showed significant changes. There was no critical dying or dead rats and no significant changes of body weight compared to the control group. In urinalysis, ophthalmic test and hematological test, there was no specific results except neutrophils and lymphocytes were significantly increased in hematological test. In the result of histopathological test, granulation tissues, vacuolated macrophages and infiltration of various inflammatory cells were distributed around the injection site.

In general, the weight of immune organs including a spleen is temporally increased by proliferation of immune cells such as T cells, B cells according to immune responses triggering by immunization. In this study, the absolute and relative spleen weight were showed significantly increased in the vaccine administrated groups compared to the control group. This result is considering as signs of activated immune responses by immunization of the test material (Charles *et al.*, 2005; Henriksen-Lacey *et al.*, 2010; Richard *et al.*, 2003) This is a serial process of immune responses triggering by vaccination and is not related toxicity.

In conclusion, the test material has not shown toxicity till the highest dose, 560 μg. It is a useful result for further studies including clinical study.

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