Lab. Anim. Res. 2010: 26(4), 369-375

Laboratory Animal Research

http://submission.kalas.or.kr

The Incidence Rate and Severity of Orthotopic Lung Cancer in an Animal Model Depends on the Number of A549 Cells and Transplantation Period

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The incidence rate of lung cancer is continually increasing, and lung cancer is the leading cause of cancer-related death worldwide. Nevertheless, few therapeutic methods are available for lung cancer. Therefore, establishing appropriate lung cancer animal models is important to investigate mechanisms and to evaluate new drugs for lung cancer. In the present study, we transplanted non-small cell lung cancer A549 human adenocarcinoma cells (2×10^4, 2.0×10^5, and 2.0×10^6 cells) into the right lobe of BALB/c nude mice via the intercostal space to develop an orthotopic lung cancer animal model that is minimally invasive and similar to human lung cancer. We then investigated the incidence rate and severity of lung cancer according to the A549 cell number (2×10^4, 2.0×10^5, and 2.0×10^6 cells) and transplantation periods (4~23 days). Lung cancer development was confirmed with gross examination, which was supported by histopathological examination. These results indicate that the incidence rate and severity of lung cancer was increased depending on the number of transplanted cells and transplantation period which the cell number and duration are increasing risk of lung cancer. Thus, this study can provide appropriate reference data to develop an orthotopic lung cancer animal model using the non-small cell lung cancer A549 cell line for researching mechanisms and evaluating candidate drugs, including various approaches for treating lung cancer.

Key words: Lung cancer animal model, orthotopic, A549

Received 7 September 2010; Revised version received 23 November 2010; Accepted 29 November 2010

Lung cancer is one of the most serious diseases worldwide with a high death rate (Jemal *et al*., 2006; Colon *et al.,* 2009). In South Korea, over 14,000 people were died in 2007 because of lung cancer (Korea National Statistical Office, 2008). Moreover, an increase in the number of people who smoke and in environmental pollutants from industries will continue to contribute to an increase in lung cancer-related death. In South Korea, the incidence rate of lung cancer has been continuously increasing (Ministry of Health and Welfare, 2009). Although stomach cancer has the highest incidence rate in South Korea, it has a relatively high survival rate. In contrast, lung cancer has a low survival rate and the highest cancer-related death rate in South Korea (Korea National Statistical Office, 2008). One reason that lung cancer has the highest death rate is that there are no effective drugs for its treatment (Tian *et al*., 2004).

Lung cancer is divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) depending on histopathological characteristics (Koizumi *et al*., 2007). The classification of lung cancer in patients is important because treatment approaches are different depend on the histopathological type of lung cancer (Hoffman *et al*., 2000). Approximately 80% of lung cancer patients reported to date had NSCLC (Määttä *et al*., 2006), and 50% of those patients had adenocarcinoma (Wakelee *et al*., 2006).

Lung cancer animal models that are similar to lung cancer in human patients are important to research the mechanism of action of lung cancer and for developing new drugs. There are several lung cancer animal models (Yamaura *et al*., 2000) such as carcinogen induced model, ectopic model, and

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orthotopic model. The orthotopic model that involves the direct transplantation of lung cancer cells into the lung parenchyma of animal is thought to be the most appropriate among several lung cancer animal models (Kang *et al*., 2006). Some scientists have already researched lung cancer using the orthotopic lung cancer animal model (Mathieu *et al*., 2004; Mijatovic *et al*., 2006).

However, the reference data for development of appropriate orthotopic model is not sufficient. For example, the incidence rate and severity of lung cancer depend on the number of cells transplanted and the transplantation period. In the present study, we investigated an appropriate orthotopic lung cancer animal model for reference data about the incidence rate and severity of lung cancer using A549 human adenocarcinoma cell lines, which have the highest incidence rate among lung cancer types in humans. We transplanted A549 cells with different cell numbers (2.0×10^4, 2.0×10^5, and 2.0×10^6) and transplantation periods (4~23 days) and then observed lung cancer development using simple, quick, and minimally invasive method. Our results suggest that the most appropriate cell number was over 2.0×10^5 and the number of transplantation day was over 15 days to achieve 100% orthotopic lung cancer animal model. This lung cancer model will be usefully used to study the disease of lung cancer and also to evaluate new drugs.

Materials and Methods

Cell lines and culture media conditions

The A549 human adenocarcinoma cell line was obtained from the Korea Cell Line Bank (Seoul, Republic of Korea). A549 cells were maintained in RPMI-1640 medium (Lonza, Basel, Switzerland) with L-glutamine supplemented with 10% fetal bovine serum (Sigma, MO, USA), 100 unit/mL penicillin, and 100 mg/L streptomycin. Cell line was maintained at exponential growth in humidified incubators at 37°C and 5% CO_2. Adherent tumor cells were harvested from cultures at 60~70% confluence by a brief exposure to trypsin and ethylenediaminetetraacetic acid (EDTA). Trypsinization was stopped with medium containing 10% serum, and cells were washed once and resuspended in serum-free medium. Trypan blue staining was used to assess cell viability and only single-cell suspensions of >95% viability were used for transplantation.

Animals

Total ninety specific pathogen free (SPF) male BALB/c nude mice, seven-week old, were obtained from Orient Bio (Kyeonggi-do, Republic of Korea) for this study. All animals were kept on a diet of Lab 5053 (PMI Nutritional International, Richmond, VA, USA) and UV-irradiated, filtered tap water. BALB/c nude mice were housed in a HEPA-filtered clean air room and kept in a temperature- (23±3°C), humidity- (50±20%), and exhaust- (10~20 exchanges/hour) controlled environment with alternating 12 h light and dark cycles. BALB/c nude mice were acclimated to these conditions for 1 week before transplantation with A549 cells (orthotopic group) or saline (control group). These experiments were approved by the Institutional Animal Care and Use Committee (IACUC) in Korea Institute of Toxicology and ethically conducted in animal facilities of Inhalation toxicology center, Korea Institute of Toxicology.

Study design of orthotopically transplanted A549 cells

A549 human adenocarcinoma cells were re-suspended in saline at a quarter volume of inoculum and mixed with three-quarter volume of growth factor-reduced matrigel (BD Bioscience, NJ, USA). Mice were anesthetized with isoflurane and restrained in the left lateral decubitus position as described previously (Cui *et al*., 2006) to transplant the A549 cells at day 0 (orthotopic group, n=5~6). Insulin syringes (0.3 mL; BD Bioscience) were used to transplant the inoculums percutaneously into the right lung parenchyma at the middle of the xiphoid process and right axilla via the intercostal space. The needle was quickly injected approximately 7 mm into the thorax; it took approximately 10 sec to transplant 30 mL of cell suspension into the BALB/c nude mice. The same volume of saline was injected into the BALB/c nude mice (control group, n=5~6). After the A549 cells were transplanted, the condition of the animals was monitored until recovery.

A different number of A549 cells (2.0×10^4, 2.0×10^5, and 2.0×10^6) was injected into each group of BALB/c nude mice (n=5~6). Lung cancer development was observed with gross and histopathological examination after necropsy at each scheduled day (Figure 1) to evaluate the effect of A549 cell number and transplantation period.

Gross and histopathologic examination of lung cancer

Mice were sacrificed using isoflurane at each scheduled day (4~23 days), and lung cancer development was observed after opening the thorax. Other organs such as the spleen, liver, kidney, and digestive organs were also observed to investigate metastatic lung cancer. The lungs were perfused intratracheally with 10% neutral buffered formalin (Biochemical, WA, USA) and fixed in 10% neutral buffered formalin. Specimens were dehydrated and embedded in paraffin, sectioned into 4 mm thick slices at intervals of 200 mm, and

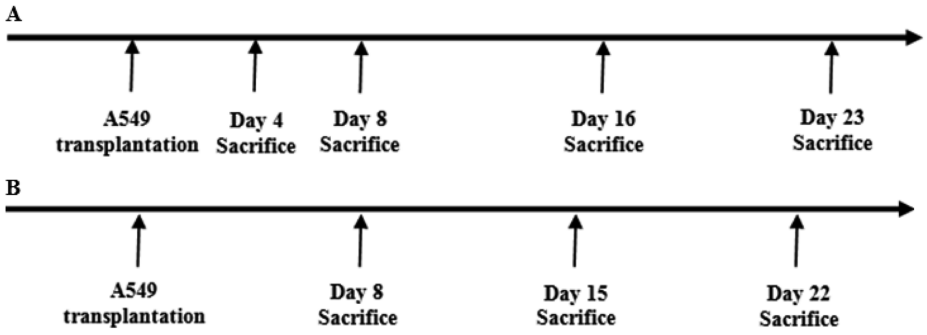

Figure 1. Study design of an orthotopic lung cancer mouse model. (A) A549 adenocarcinoma cells (2.0×10^4) were transplanted into BALB/c nude mice. (B) A549 adenocarcinoma cells (2.0×10^5 and 2.0×10^6) were transplanted into BALB/c nude mice.

stained with hematoxylin and eosin (H&E) for histopathological examination. Among the histopathological slides of the orthotopic group, 3 were selected with severe cancer development, and the total area of lung cancer was calculated using the DIXI Imaging solution (Pharmtech, Kyeonggi-do, Republic of Korea) software program to quantify the severity of lung cancer.

Statistical analysis

Results were expressed as mean±SE for analysis of tumor area differences depend on A549 cell number and transplantation days between the groups. Variance of numerical data was checked by the Levence's test. If the variance was homogeneous, the data was analyzed with the two-way ANOVA method to compare the statistical significance (SPSS 12.0, SPSS Incorporation, NY, USA). Statistical significance was established when $P<0.05$.

Results

Clinical signs and lung cancer development

There were no characteristic clinical signs or cancer-related death during the experimental period. The initiation of lung cancer development was observed with gross examination during necropsy at day 8. However, there was no evidence of metastasis to other major organs during the experimental period. After gross examination, lung cancer development was examined histopathologically using a microscope, which confirmed lung cancer development (Figure 2). Histopathological lung cancer development was observed from day 4 after transplantation of A549 cells.

Incidence rate and severity of lung cancer depends on A549 cell number

After gross and histopathological examination, we concluded that the increase in the lung cancer incidence rate was dependent on cell number (Table 1). In the group transplanted with 2.0×10^4 or 2.0×10^5 A549 cells, lung cancer development was observed by gross examination at day 8 in 2 of 5 or 3 of 6 animals, respectively. Gross examination of the group transplanted with 2.0×10^6 A549 cells indicated that the lung-cancer incidence rate was the highest (4 of 6 animals). Histopathological observation indicated that in the group transplanted with 2.0×10^4 A549 cells, lung cancer development

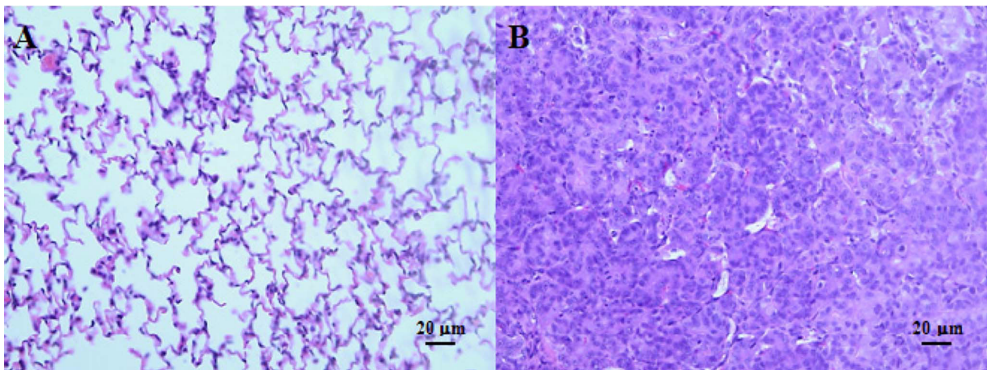

Figure 2. Histopathological observation of the lung tissue. (A) Saline was injected into BALB/c nude mice at day 22. (B) A549 adenocarcinoma cells (2.0×10^6) were transplanted into BALB/c nude mice at day 22.

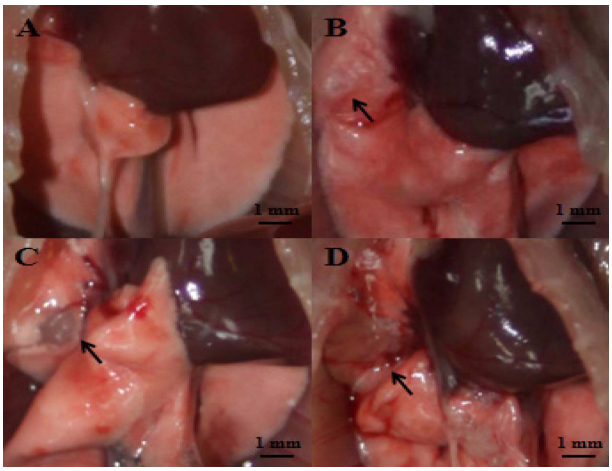

Figure 3. Gross examination after transplantation of A549 adenocarcinoma cells, indicates that the severity of lung cancer (arrow) depends on the number of transplanted cells. (A) Saline was injected into BALB/c nude mice at day 22. (B) A549 adenocarcinoma cells (2.0×10^4) were transplanted into BALB/c nude mice at day 23. (C) A549 adenocarcinoma cells (2.0×10^5) were transplanted into BALB/c nude mice at day 22. (D) A549 adenocarcinoma cells (2.0×10^6) were transplanted into BALB/c nude mice at day 22.

was observed in 4 of 5 animals at day 4; all transplanted animals developed lung cancer after day 8. Both the incidence rate and severity of lung cancer development observed by gross examination and histopathologically was dependent on transplanted A549 cell number (Figures 3 and 4).

Incidence rate and severity of lung cancer depends on the number of A549–cell transplantation days

The increase in the lung cancer incidence rate was dependent on the number of transplantation days (Table 1). In the group transplanted with 2.0×10^4 A549 cells, gross examination indicated that there were 2 of 5, 3 of 5, and 5 of 6 animals that developed lung cancer at day 8, day 16, and day 23, respectively. A similar trend in the increase in lung cancer incidence rate with transplantation period was observed in the groups transplanted with 2.0×10^5 and 2.0×10^6 A549 cells. Taken together, our data indicate that the severity of lung cancer development was dependent on the number of transplanted A549 cells and transplantation period (Figure 5).

Quantification of lung cancer severity

The severity of lung cancer was quantified to compare the effect of A549 cell number and number of transplantation days (Figure 6). These results suggest that the severity of lung

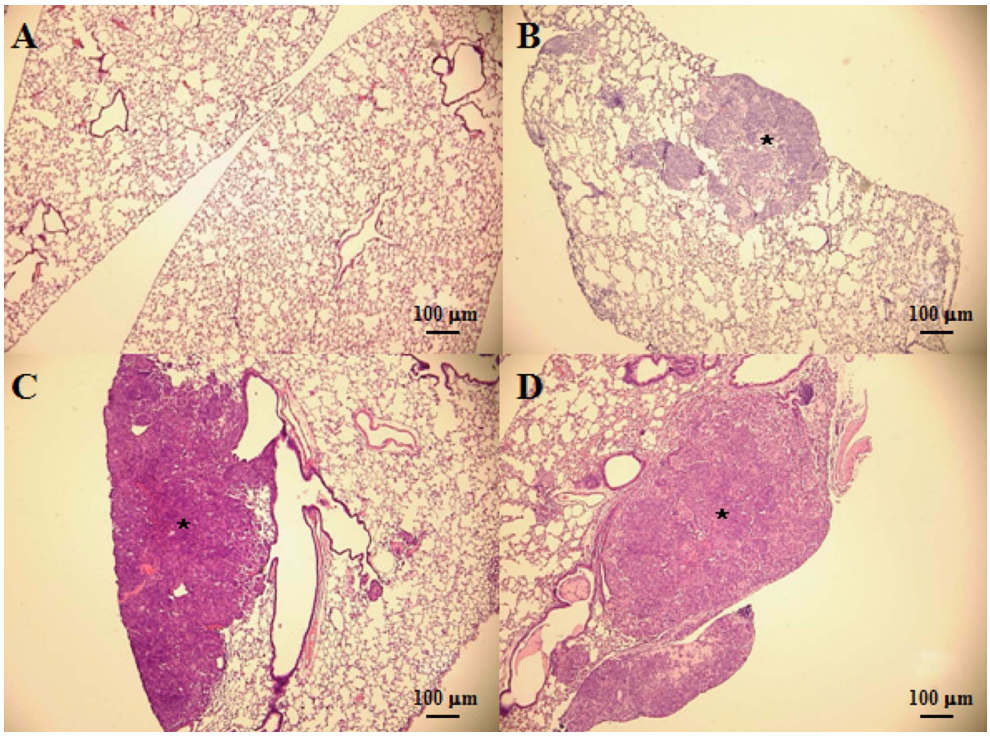

Figure 4. Histopathological examination after transplantation of A549 adenocarcinoma cells indicates that the severity of lung cancer (star) depends on the number of transplanted cells. (A) Saline was injected into BALB/c nude mice at day 22. (B) A549 adenocarcinoma cells (2.0×10^4) were transplanted into BALB/c nude mice at day 23. (C) A549 adenocarcinoma cells (2.0×10^5) were transplanted into BALB/c nude mice at day 22. (D) A549 adenocarcinoma cells (2.0×10^6) were transplanted into BALB/c nude mice at day 22.

Table 1. The incidence rate of lung cancer observed with gross and histopathological examination depends on the number of A549 adenocarcinoma cells

Examination	Gross				Histopathological			
Day / Cell amount	Saline	[a]2.0×10^4	[b]2.0×10^5	[b]2.0×10^6	Saline	[a]2.0×10^4	[b]2.0×10^5	[b]2.0×10^6
Day 4	0/5	0/5	-	-	0/5	4/5	-	-
Day 8	0/5	2/5	3/6	4/6	0/5	5/5	6/6	6/6
Day 15	0/6	-	6/6	6/6	0/6	-	6/6	6/6
Day 16	0/5	3/5	-	-	0/5	5/5	-	-
Day 22	0/6	-	6/6	6/6	0/6	-	6/6	6/6
Day 23	0/6	5/6	-	-	0/6	6/6	-	-

[a]Gross and histopathological observation was performed after necropsy at day 4, 8, 16, and 23.
[b]Gross and histopathological observation was performed after necropsy at day 8, 15, and 22.

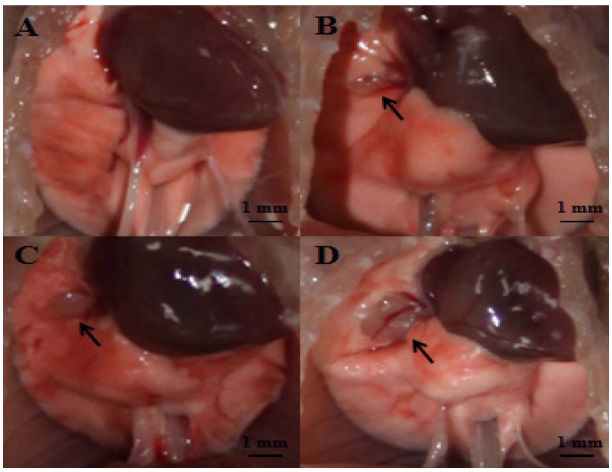

Figure 5. Gross examination after transplantation of A549 adenocarcinoma cells indicates that the severity of lung cancer (arrow) depends on the number of transplantation days. (A) Saline was injected into BALB/c nude mice at day 22. (B) A549 adenocarcinoma cells (2.0×10^6) were transplanted into BALB/c nude mice at day 8. (C) A549 adenocarcinoma cells (2.0×10^6) were transplanted into BALB/c nude mice at day 15. (D) A549 adenocarcinoma cells (2.0×10^6) were transplanted into BALB/c nude mice at day 22.

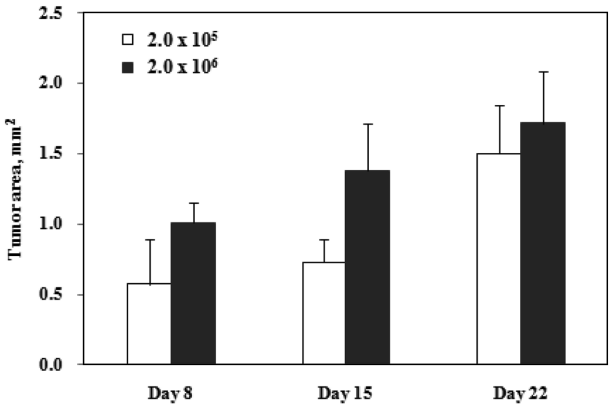

Figure 6. Tumor area in histopathological slides depends on the number of transplanted A549 adenocarcinoma cells and transplanted days (mean±SE). Tumor area depends on transplanted days was significantly different ($P<0.05$). n=5~6.

cancer was gradually increased depending on the number of transplanted A549 cells and transplantation period. Especially, tumor area was statistically different ($P<0.05$) depends on A549-cell transplantation days. These data correlate with the gross and histopathological data showing that the severity of lung cancer development was dependent on the number of transplanted A549 cells and the transplantation period.

Discussion

Lung cancer is one of the most serious diseases worldwide, including South Korea. Therefore, many researchers have tried to elucidate the mechanism of lung cancer progression and to develop more effective lung cancer drugs. To that end, appropriate lung cancer animal models that are similar to human lung cancer in patients need to be developed. Greater similarity between these models and human lung cancer will provide more relevant results from lung cancer animal models. Several lung cancer animal models have been developed; the most widely used animal models are the carcinogen-induced lung cancer model using urethane (Stathopoulos *et al.*, 2007; Minowada and Miller, 2009), the ectopic lung cancer model (Mazieres *et al.*, 2004; Hassid *et al.*, 2006) that subcutaneously transplants lung cancer cells into the flank of animals, and the orthotopic lung cancer model (Mijatovic *et al.*, 2006; Cai *et al.*, 2008) where the cancer is developed directly in the lung tissue as in human lung cancer. Until now, many lung cancer-related researchers have used the ectopic model. However, this model has been criticized since there are differences between lung cancer in patients and the ectopic lung cancer model (Onn *et al.*, 2003). For example, differences in the location of the cancer (flank) versus the lung, and the cancer in the ectopic model does not metastasize as in human patients (Manzotti *et al.*, 1993). Therefore, the

relevance of the orthotopic model has increased. There are 3 types of orthotopic models according to the transplantation method of injection: via the trachea (McLemore *et al*., 1987), via the tail vein (Goto *et al*., 2002), and direct transplantation into the parenchyma of the lungs (Doki *et al*., 1999). Transplantation of lung cancer cells into the parenchyma of the lungs is the most non-invasive method and most similar to human lung cancer.

There are previous reports of an induced orthotopic lung cancer animal model describing the transplantation of A549 cells into the parenchyma of the lungs (Onn *et al*., 2003; Mathieu *et al*., 2004). The results of these studies described only the survival period of A549-transplanted nude mice. Onn *et al*. (2003) transplanted 1.0×10^6 A549 cells and the survival period was 50~60 days. Mathieu *et al*. (2004) transplanted 2.0×10^6 A549 cells and the survival period was 30~50 days. These data are not enough to develop an orthotopic lung cancer model for various purposes. In this study, we investigated the dependence of the incidence rate and severity of lung cancer development on the number of transplanted A549 cells (2.0×10^4, 2.0×10^5, and 2.0×10^6) and the transplantation period (4~23 days) to provide reference data for orthotopic model development. We also observed lung cancer-related death at approximately day 90 after transplantation of 2.0×10^6 A549 cells (n=2, data not shown). The thoracic cavity was filled with multiple lung cancer nodules after necropsy, which suggests that the lung cancer was continuously proliferating as indicated at day 4~23. Another report by Kraus-Berthier *et al*. (2000) transplanted 1.0×10^6 A549 cells into the thoracic cavity, not lung parenchyma; however, a large variation in the survival period was observed (21~86 days).

In this study, we concluded that the increase in the incidence rate and severity of lung cancer development was dependent on the number of A549 cells and transplantation period on the basis of data from our simple, quick, and minimally invasive lung cancer model. And we also confirmed that over 2.0×10^5 of cell number and over 15 days of transplantation day were the appropriate condition to attain 100% orthotopic lung cancer animal model. Furthermore, these results provide some reference data for the development of an A549-induced orthotopic lung cancer animal model since we provide information about the establishment of A549 cells in a transplantation orthotopic model that can be used for other study purposes. Moreover, this animal lung cancer model is similar to human lung cancer and can be used to study the mechanism of lung cancer progression and to develop new anti-cancer drugs.

Acknowledgments

This study was supported by the Ministry of Knowledge Economy for General Project grant of the establishment of the International Inhalation Toxicology Evaluation technology of Korea Institute of Toxicology.

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