

Recovery from the Two-generation Reproductive Toxicity in Sprague-Dawley Rats by Treatment with Decursin and Decursinol Angelate

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The aim of this study was to determine the effect of decursin (D) and decursinol angelate (DA) against bisphenol A (BPA) toxicity in a rat two-generation study. Adult rats were divided into the following three groups: (1) control, (2) BPA, and (3) BPA+D/DA. The D and DA treatment of F0 parents increased the terminal body weight and relative adult organ weights (testes, kidneys, spleen, and liver) when compared with the BPA group. A significant decrease in sperm count was found in the BPA+D/DA (7.69%) and BPA (64.70%, $p < 0.01$) groups, when compared with the sperm count in the control group. No offspring were obtained in the F1 generation of the BPA (50 mg/kg/day) group, but the addition of D/DA in the BPA+D/DA group significantly restored fertility (55.78%) and gestation indices (98.87%) in the F1 generation. No significant differences were found in the fertility index between the control (75.02%) and the BPA+D/DA (78.11%) groups in the two-generation study, when compared with the one-generation study. The viability ratio during lactation in the D/DA group was also similar to that of the control group. These data indicate that D/DA (50 mg/kg/day) administered over two generations causes significant positive changes in reproductive or developmental parameters.

Key words : *Angelica gigas* Nakai, decursin, decursinol angelate, reproductive toxicity, two-generation

Introduction

Epidemiological data showed that the human semen quality has declined during the last 60 years, whereas the incidence of male genital tract abnormalities and infertility has increased. There has been increased awareness of the possible effects of environmental contaminants on male reproduction. BPA, a potential endocrine disruptor and testicular toxicant, also has been shown to affect the reproduction of male rats and mice [19, 21]. The chemical structure of BPA is similar to the more potent estrogenic compound diethylstilbestrol and has been shown to mimic estradiol action by stimulating testicular toxicity of BPA at high doses in rats and mice [5].

Angelica gigas Nakai (*A. gigas*) is a Korean traditional herbal medicine that is one of the most popular herbal medicines used in Asian countries, including Korea, Japan and China. *A. gigas* is also marketed as a functional food prod-

uct in Europe and America. *A. gigas* of Korea is quite distinct in that it has deep purple flowers while Japan and China *Angelica* have white flowers [1]. *A. gigas* has been studied extensively and found to contain a variety of substances including coumarins [6, 17]. Coumarins are comprised of decursin and decursinol angelate (D/DA), which has been used as a traditional medicine for the treatment of anemia, as a sedative, and as an anodyne or a tonic agent [24]. Previously, we reported the oral acute and subacute and the genotoxicity in D/DA having the content of about 95% [9, 10]. In addition, *A. gigas* has been widely used for the treatment of dysmenorrhea, amenorrhea, menopausal syndromes, abdominal pain, injuries, migraine headaches and arthritis. Furthermore, *A. gigas* is known to exert antibacterial and anti-amnesic effects as well to induce inhibitory effects against acetylcholinesterase, depression of cardiac contraction, and activation of protein kinase C [2, 7, 13, 14, 25]. Several herbs that were used in traditional medicine demonstrated antioxidant or free radical scavenging activities [8]. Further, some of the herbal supplements with antioxidant properties exhibited a beneficial effect on the sperm function. These showed antioxidant activities in various diseases besides male infertility causing decreased sperm quality [8].

We have found that D/DA have a new effect on increas-

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ing sperm cells [11]. In this study, we studied the effects of exposure of rat to D/DA on reproduction and development in a two-generation reproductive toxicity study.

Materials and Methods

Chemicals and reagents

Isolation and purification of D/DA in *A. gigas* Nakai were performed as described previously [12]. D/DA was extracted from *A. gigas*, and HPLC analysis revealed that it had a purity of 75% [11]. BPA, estradiol benzoate, and progesterone were obtained from Sigma (St. Louis, MO, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA), and the solvents used were of analytical grade.

Animal studies

Male and female Sprague-Dawley (SD) rats at 4 weeks of age were used in this experiment. Experimental protocol (number: 2010-003A) was approved by the Institutional Animal Care and Use Committee of the Kyungshung University. Prior to each experiment, the animals were fasted overnight but were provided with free access to water. They were kept in a temperature-controlled environment ($23\pm 2^{\circ}\text{C}$) with a 12-hr light-dark cycle and provided with food and water ad libitum. Bioassays were conducted according to the World Health Organization guideline for evaluation of the safety and efficacy of herbal medicines [23]. The animals were divided into a control group and two treatment groups, each consisting of ten for males and thirty for females. The control group received sterilized water while the treated groups received 50 mg/kg body weight (D/DA) of the recovered concentration of sperm counts in acute toxicity study [12] by gavage and 50 mg/kg body weight (highest dose of BPA) in the range described as Tyl *et al.* [21] by peritoneal injection for 7 days. BPA and D/DA were dissolved in absolute ethanol and then mixed with corn oil to final absolute ethanol concentration of 0.5% (a dose volume of 5 ml/kg body weight). The BPA+D/DA group received D/DA solution (only male rats) by gavage for 7 days after receiving BPA solution by peritoneal injection for 7 days prior to the mating period. The BPA group received BPA solution (only male rats) peritoneal injection for 7 days prior to the mating period. Estradiol benzoate and progesterone were also administered by subcutaneous injection at 50 $\mu\text{g}/\text{kg}$ and 750 $\mu\text{g}/\text{kg}$ (only female rats

of all groups) before 48 hr and 4 hr for the successful mating [15]. After the last treatment day, the rats were sacrificed and the testes and other organs were dissected. The organs were then washed with saline (0.9% NaCl; w/v) and weighed. The relative weight of the organs (average weight of the organs) was expressed in terms of g/100 g of body weight. The right testis of each rat was used for sperm analysis.

Mating procedures and sperm analysis

The mating periods for F0 males ($n=10$) and females ($n=30$) was 2 weeks. Also we have two-generation testing by mating within one-generation group (the control and BPA+D/DA groups of F1 males ($n=10$) and females ($n=30$)). One-litter from one-mother has a dwarfism from one-generation, and then we want to know about their baby size in two-generation state. Among one-generation, 10 male rats were used for testing in two-generation. All generation babies were evaluated by the number of pups of litter, fertility index, gestation index, and viability index after maintaining with normal condition. During mating period, vaginal smears were identified for the presence of sperm. The first day of pregnancy was designated from the day of successful mating. All adults were observed for clinical signs of toxicity and body weights. Body weights of females were recorded for pregnancy and lactation exhibiting evidence of successful mating. For the effects of D/DA against BPA, body weights and organ weights of F1 pups (only males) were evaluated and sacrificed. Sperm analysis was determined as previously the method [11]. The epididymis was dissected and immediately minced with fine scissors in 20 ml of physiological saline (0.9% NaCl) at 37°C and then incubated at 37°C for 30 min to allow spermatozoa to leave the epididymal tubules. Approximately 10 μl of sperm suspension was placed on a hemocytometer for light microscope observation. The counting of both motile and immotile sperms was done using a hemocytometer. Sperm motility was expressed as a percentage of motile sperm of the total sperm counted. Daily sperm production per gram of testis were calculated according to the method described by Akhtar *et al.* [3].

Statistical analysis

Data were expressed as the means \pm standard deviation (S.D.). Statistical analyses were performed using the Sigma Plot 10.0 program. Values were compared to control by per-

forming analysis of variance (ANOVA) followed by unpaired Student's *t*-test. *p* values <0.05, 0.01, and 0.001 were considered significant.

Results and Discussion

Preclinical observations, body weight during pre-mating, mating, gestation, and lactation periods

A two-generation toxicity study was performed to evaluate the recovered effects of D/DA on reproduction and development in rats. Adult systemic toxicity was evident for F0 (parent) and F1 (offspring) retained rats at 50 mg/kg/day of BPA and BPA+D/DA groups, including consistent reductions in body weights in F0 and F1 generations (Table 1). There were no major influences of toxicity in F0 and F1 rats during pre-mating, mating, gestation, or lactation period in BPA+D/DA group. Body weights for F0 and F1 rats are presented in Fig. 1. There were decreased effects ($p < 0.01$) of BPA and BPA+D/DA treatment on F0 males and females body weights during 1-week prebreed period, the 2-week mating period, or the postmating period. Similar

results were recorded by Tyl *et al.* [21], who mentioned that BPA (50 mg/kg/day) decreased the body weight in SD rats. The body weight and body weight gain of F1 male exhibited significant differences between BPA+ D/DA and control groups. However, there was no offspring for F1 generation in the high dose of BPA group. In the BPA+D/DA group, final F1 male (36.59%, $p < 0.001$) body weight decreased significantly compared to the corresponding weights in the control group (Fig. 1). Body weights at sacrifice were significantly decreased ($p < 0.01$) in all generations (Table 1). At BPA+D/DA group, reduced food consumption was observed in the offspring. A significant decrease in 7 days and mating period was observed at BPA+D/DA group in F1 males (data not shown). However, there was no difference in BPA+D/DA and control groups on F2 (grandoffspring) males body weights throughout pre-mating, mating, gestation, or lactation period except for one litter from one-mother. The data indicate that doses of D/DA in F0 males recover reproductive or developmental parameters in rats.

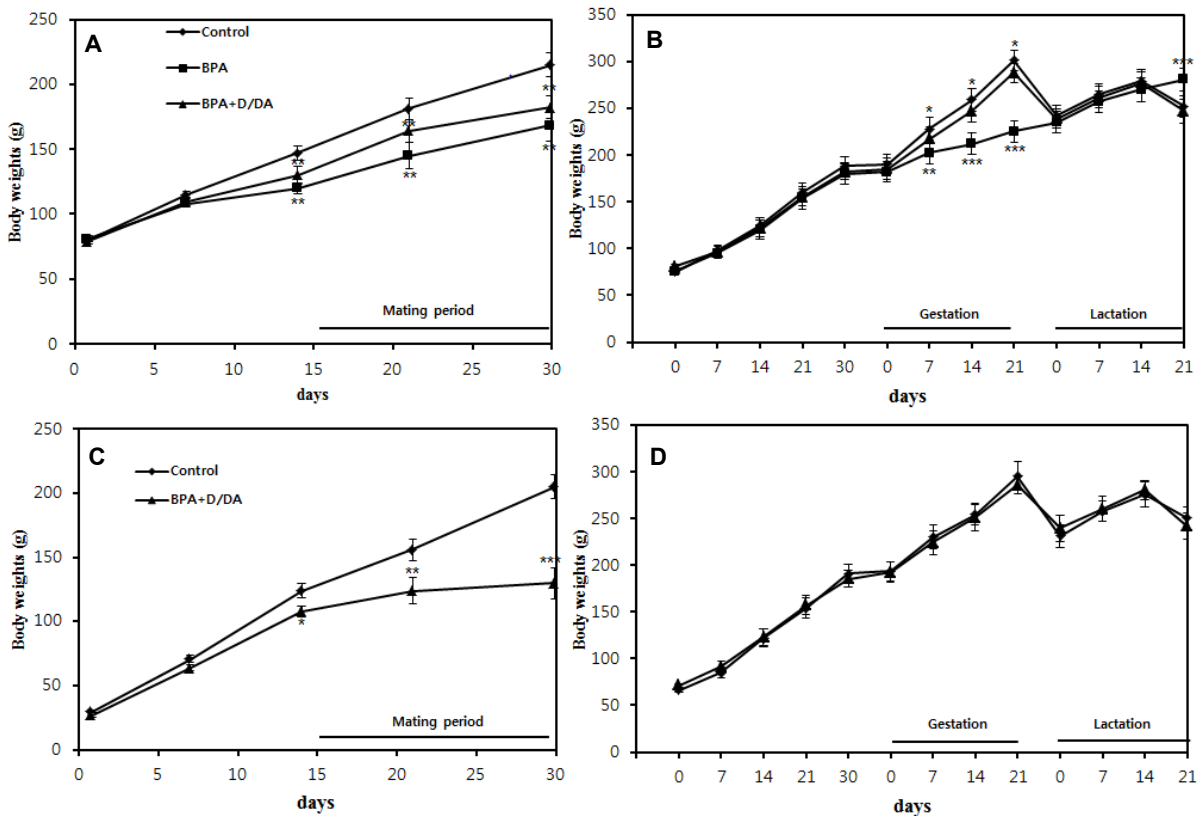


Fig. 1. Body weight of F0 males (A, n=10), F0 females (B, n=30), F1 males (C, n=10) and F1 females (D, n=30). Significantly different from the control, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 1. Absolute and relative organ weights of males (F0, F1, and F2) were administrated for 30 days

Weights	Generation	Group		
		Control	BPA	BPA+D/DA
Initial body weight (g)	F0	80.02±1.32	81.05±2.00	79.11±1.91
	F1	24.08±0.23	-	30.47±0.31
	F2	35.01±1.50	-	32.47±1.81
Terminal body weight (g)	F0	215.01±9.51	168.12±12.21	182.23±8.92
	F1	205.12±9.24	-	130.02±9.17
	F2	215.47±10.87	-	207.59±9.98
Absolute organ weight (g)				
Testes (g)	F0	2.31±0.11	1.98±0.24	2.05±0.28
	F1	2.29±0.32	-	1.60±0.12
	F2	2.28±0.11	-	2.12±0.78
Epididymides (mg)	F0	657.01±54.02	624.02±66.05	668.91±70.21
	F1	668.28±32.12	-	417.49±69.19
	F2	647.28±41.11	-	632.97±54.71
Preputial glands (mg)	F0	151.23±12.12	127.79±21.54	148.98±10.37
	F1	152.74±14.87	-	87.74±19.11
	F2	148.85±11.74	-	145.32±9.87
Liver (g)	F0	8.59±1.21	7.21±0.88	7.67±0.70
	F1	9.57±1.74	-	6.42±0.51
	F2	9.25±0.98	-	9.11±0.22
Kidney (g)	F0	1.54±0.11	1.32±0.08	1.46±0.18
	F1	1.42±0.07	-	0.98±0.11
	F2	1.47±0.04	-	1.39±0.08
Spleen (mg)	F0	462.33±12.74	401.11±24.56	421.55±23.77
	F1	432.11±13.79	-	311.94±19.87
	F2	458.99±11.12	-	445.85±15.71
Relative organ weight (per 100 g body)				
Testes (g)	F0	1.07±0.05	1.17±0.12*	1.12±0.13* ¹⁾
	F1	1.11±0.16	-	1.23±0.05*
	F2	1.05±0.01	-	1.02±0.29
Epididymides (mg)	F0	306.08±25.12	315.11±33.34	316.67±33.24
	F1	325.80±15.66	-	321.10±53.21
	F2	300.40±19.44	-	304.91±24.99
Preputial glands (mg)	F0	70.34±5.64	64.50±10.87	70.52±4.91
	F1	74.46±5.85	-	67.48±7.80
	F2	69.08±4.97	-	70.01±4.01
Liver (g)	F0	3.99±0.56	4.28±0.44**	4.20±0.33*
	F1	4.66±0.68	-	4.93±0.21*
	F2	4.29±0.01	-	4.38±0.09
Kidney (mg)	F0	716.25±0.05	785.15±0.04**	801.19±0.09** ²⁾
	F1	692.28±0.03	-	753.53±0.08**
	F2	682.22±0.01	-	669.59±0.03
Spleen (mg)	F0	215.02±5.93	238.58±12.40**	231.32±11.25**
	F1	210.66±5.43	-	239.91±8.11**
	F2	213.01±4.98	-	214.77±6.91

All values represent the means±S.D. (n=15). ¹⁾²⁾Significantly different from the control.* $p<0.05$, ** $p<0.01$.

Organ weights in F0, F1, and F2 generation

Previously, we reported the effect of organ weights by BPA and D/DA [11]. A significantly decreased weight of the body weight, testes, liver, kidney and spleen was de-

tected in F0 and F1 males at 50 mg/kg/day of BPA+D/DA. The organ weights of F0 and F1 males are presented in Table 1. Although the absolute weights of the Epididymides and the Preputial glands at BPA were significantly lower,

no significant differences in the relative weights between the control and BPA groups. However, there was consistently affected in F1 offspring in BPA group. In the BPA+D/DA group, the terminal body weights of F0 (17.98%, $p<0.05$) and F1 (57.76%, $p<0.01$) decreased and the weights of the testes F0 (4.47%, $p<0.05$) and F1 (8.76%, $p<0.05$), the liver F0 (5.00%, $p<0.05$) and F1 (5.48%, $p<0.05$), the kidney F0 (10.61%, $p<0.01$) and F1 (8.13%, $p<0.01$), the spleen F0 (7.05%, $p<0.01$) and F1 (12.20%, $p<0.01$) increased significantly compared to the corresponding relative weights in the control group. Absolute and relative organ weight data for F1 weanling pups indicate that the absolute organ weights were reduced, and the relative weights were increased in BPA+D/DA group. Some of the effects on weights in the F1 weanlings were also present in the adults. These results may cause the effect by reduced terminal body weight at dietary dose. However, some of the effects on absolute weights in the F0 males were significantly reduced at only BPA group compared to the BPA+D/DA group. Similar results were also recorded by Tyl *et al.* [21], who mentioned that the highest dose of BPA intakes approximately 1.5 to 2 times in the food consumption compared to other doses. These data indicated that the weights of the kidney, liver, spleen and testes were significantly correlated ($p<0.05$ and $p<0.01$) with body weight. The organ weight of F2 males are presented in Table 1. No significant differences in the weight of any organs between control and BPA+D/DA groups were detected in F2 males. These results indicate that oral doses of 50 mg/kg/day of D/DA recover damaged reproductive parameters by BPA over two-generation. More importantly, although organ weights of F0 and F1 males in the BPA+D/DA and BPA groups were different from those in the control group, the evidence of organ weights by D/DA (F2 males) affected, in as much as the overall ANOVA showed significant differences ($p<0.05$ and $p<0.01$) between the BPA+D/DA and BPA groups.

Epididymal sperm analysis and reproductive effects

It is known that administration of D/DA increases the epididymal sperm motility and sperm count by the antioxidant effect of lipid peroxidation when compared with the corresponding group of control animals [20]. The mean epididymal sperm number was decreased (64.70%, $p<0.01$) in the BPA group compared to that of the control group. However, the mean epididymal sperm number was slightly decreased (7.69%, $p<0.05$) in the BPA+D/DA group com-

pared to that of the control group. These results match those observed in our earlier studies which D/DA recover sperm cell count against BPA damage [11]. The reproductive parameters for F0 parent/F1 offspring are shown Table 2. There were significant differences in the fertility index and gestation index during lactation of BPA group. Although the BPA group was observed copulation of males and females, no significantly pups were observed in BPA group. The majority of the effects observed for parental reproductive parameters (the epididymal sperm concentration of males) occurred only at the highest dose of BPA in BPA group. Tyl *et al.* [21] demonstrated that the highest dose of BPA was significantly reduced the number of implants, total pups, and live pups per litter at birth. Biegel *et al.* [4] and Liu *et al.* [15] also suggested that BPA may be reduced live litter sizes associated with reduced number of implantations per litter and a major contributor leading to male reproductive disorders. Reel *et al.* [18] also concluded that BPA was a reproductive toxicant that caused a reduction in the number of live pups born in the F0 generation, reduced sperm motility and weight of some male reproductive organs in both the F0 and F1 generation, and reduced postnatal survival of the F1 generation. In the BPA+D/DA group, fertility index (55.78%, $p<0.05$) and gestation index (98.87%) restored significantly compared to the corresponding in the BPA group (Table 2). Previously, we reported that D/DA acts as protective agents against BPA-induced testicular toxicity. Administration of D/DA with BPA treatment also restored the testicular damage, and quality of sperm [11]. McLachlan *et al.* [16] suggested that a change of sperm count was directly affected to a spermatogenesis, including a level of testosterone. The luteinizing hormone of the pituitary gland regulated the secretion of testosterone, lead to a difference of spermatozoa [22]. Our results also showed a change of testosterone level (data not shown). As shown in Table 2, an increase in the level of fertility index, gestation index and viability index during lactation were observed in reproductive parameters of rats exposed to BPA+D/DA compared to BPA group. The fertility index (22.24%) and gestation index (0.27%) in the BPA+D/DA group were a little lower when compared with control and BPA+D/DA groups. The reproductive parameters for F1 parent/F2 offspring are also shown in Table 2. The fertility index of control (75.02%) and BPA+D/DA (78.11%) groups were no significant difference for two-generation compared to one-generation. This result in-

Table 2. Reproductive data for F0 parent / F1 offspring and F1 parent / F2 offspring

	Group		
	Control	BPA	BPA+D/DA
F0 parent / F1 offspring			
No. of pairs	20	20	20
Precoital interval (days)	15.01±0.8	15.02±0.6	15.01±0.9
Copulation index (%) ¹⁾			
Male	100.00	100.00	100.00
Female	100.00	100.00	100.00
Fertility index (%) ²⁾	78.02	0** ⁹⁾	55.78* ⁸⁾
Gestation index (%) ³⁾	99.14	0**	98.87
Sex ratio of pups ^d	0.54	-	0.48
Viability index during lactation			
Day 0 ⁴⁾	100.00	-	100.00
Day 7 ⁵⁾	99.23	-	97.98
Day 21 ⁶⁾	98.19	-	97.53
F1 parent / F2 offspring			
No. of pairs	20	-	20
Precoital interval (days)	14.98±0.2	-	15.02±0.3
Copulation index (%) ¹⁾			
Male	100.00	-	100.00
Female	100.00	-	100.00
Fertility index (%) ²⁾	75.02	-	78.11
Gestation index (%) ³⁾	95.22	-	96.12
Sex ratio of pups ⁴⁾	0.55	-	
Viability index during lactation			0.58
Day 0 ⁵⁾	100.00	-	100.00
Day 7 ⁶⁾	99.58	-	99.37
Day 21 ⁷⁾	99.44	-	99.29

¹⁾Copulation index (%)= (No. of rats copulated/no. paired) ×100.

²⁾Fertility index (%)= (No. of females pregnant/no. of females copulated) ×100.

³⁾Gestation index (%)= (No. of females with parturition/no. of females pregnant) ×100.

⁴⁾Sex ratio pups= No. of male pups/total no. of pups.

⁵⁾Viability index on day 0 of lactation (%)= (No. of live pups delivered/total no. of pups delivered) ×100.

⁶⁾Viability index on day 7 of lactation (%)= (No. of live pups delivered/total no. of pups delivered) ×100.

⁷⁾Viability index on day 21 of lactation (%)= (No. of live pups delivered/total no. of pups delivered) ×100.

Significantly different from the control. ⁸⁾* $p < 0.05$, ⁹⁾** $p < 0.001$.

icates that D/DA affect reproductive performance in generation. No significant difference in the fertility index, gestation index, and viability index during lactation was also observed between control and BPA+D/DA groups. In our study, these findings suggest that D/DA effects on the number of pups per litter, sex ratio, fertility index, gestation index, and viability index in rats exposed to BPA (50 mg/kg/day) during lactation. Animals treated with D/DA increased the number of mating and mounting comparing with animals treated with only BPA. Fertility index and body size of animals treated with D/DA were also normalized in two-generation. Though every baby was normalized in two-generation, further extensive research will be needed

for exacted evaluation in several conditions.

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초록 : Decursin and decursinol angelate가 2세대 출산률 회복에 미치는 영향

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2세대동물에 있어 bisphenol A (BPA)에 대하여 decursin and decursinol angelate (D/DA)의 투여가 출산률 회복에 미치는 영향에 대하여 연구하였다. 실험그룹은 대조군, BPA 투여군, BPA와 D/DA 병용투여군으로 이루어졌으며, BPA와 D/DA 병용투여군에서 BPA 투여군과 비교 시 어미개체에서 몸무게, 장기무게에서 차이를 보였다. 정자수는 대조군과 비교 시 BPA군에서는 64.70%로 감소하였고 BPA와 D/DA 병용투여군에서는 7.69% 감소를 확인하였다. 고용량의 BPA (50 mg/kg/day)의 복용개체에서는 1세대에 임신 및 출산을 하지 못하였다. 하지만, BPA와 D/DA 병용투여군에서는 BPA 투여군과 비교 시 임신(98.87%) 및 출산률(55.78%)이 회복 됨을 확인하였다. 또한, 2세대의 임신 및 출산률은 대조군(75.02%)과 비교 시 BPA와 D/DA 병용투여군(78.11%)에서는 차이가 없음을 확인하였다. 이 연구결과는 D/DA (50 mg/kg/day)의복용은 임신 및 출산에 있어 긍정적인 영향을 주는 것으로 확인하였다.