Effect of lithospermate B on spermatogenesis in aroclor 1254 exposed C57BL6 mice

Daham Kim

Department of Medicine

The Graduate School, Yonsei University

Effect of lithospermate B on spermatogenesis in aroclor 1254 exposed C57BL6 mice

Directed by Professor Eun Jig Lee

The Master's Thesis
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Master of Medical Science

Daham Kim

December 2011

This certifies that the Master's Thesis of
Daham Kim is approved.

Thesis Supervisor : Eun Jig Lee

Thesis Committee Member#1 : Nam Hoon Cho

Thesis Committee Member#2 : Young Deuk Choi

The Graduate School
Yonsei University

December 2011

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who gave me the possibility to complete this thesis.

First of all, I am deeply indebted to my supervisor, Professor Eun Jig Lee, whose stimulating suggestions and encouragement helped me throughout my academic life.

I wish to express my warm and sincere thanks to Professor Nam Hoom Cho, and Young Deuk Choi who advising and encouraging me throughout my years at the graduate school.

I would like to thank my laboratory colleagues for their assorted efforts and supports.

Finally, I especially want to thank my family for their loving support.

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Effect of lithospermate B on spermatogenesis
in aroclor 1254 exposed C57BL6 mice

Daham Kim

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Eun Jig Lee)

Background: Polychlorinated biphenyls (PCBs) are known to induce oxidative stress in the reproductive organs and some antioxidants showed protective effects. Lithospermate B (LAB) showed antioxidant effects in some previous studies. We tested whether LAB can exhibit a similar protective effect on testicular injury induced by PCB.

Methods: Adult C57BL6 mice were divided into three groups. First group received corn oil and normal saline as vehicle for 30 days. Second group received Aroclor 1254 at a dose of 2 mg/kg per day and normal saline for 30 days. Third group received Aroclor 1254 at a dose of 2 mg/kg per day and LAB at a dose of 20 mg/kg per day for 30 days. 24 hours after last treatment, testes and epididymides were removed. Sperms were collected from the epididymis and its count was calculated. After homogenized testis, activities of antioxidant enzymes (SOD, CAT, GPx, GR) and levels of ROS (H_2O_2) were estimated.

Results: Although there were no statistically significant differences, the result of this experiment shows that PCB decreases sperm density, activities of antioxidant enzymes and increases levels of ROS. Supplementation of LAB seemed to restore these parameters.

Conclusion: In conclusion, LAB has protective effect by enhancing of antioxidant enzyme activities against testicular injury induced by PCB.

Key words : lithospermate B, spermatogenesis, aroclor 1254

Effect of lithospermate B on spermatogenesis
in aroclor 1254 exposed C57BL6 mice

Daham Kim

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Eun Jig Lee)

I. INTRODUCTION

Polychlorinated biphenyls (PCBs), members of environmental contaminants, belong to the halogenated hydrocarbon class. PCBs are biomagnified along food chains, increasing a risk of human exposure due to the ubiquitous, persistent and lipophilic characters. Several adverse effects have been documented in animals due to PCB exposure on different organs and at different metabolic levels such as the thyroid hormone metabolism and gonadal steroidogenesis and semen quality. [1, 2]

Aroclor 1254, a commercial mixture of PCBs, contains 54% chlorine by weight. Previous studies showed in adult male rats that Aroclor 1254 induced an increase in the lipid peroxidation, hydrogen peroxide and hydroxyl radical and diminish in the antioxidant defense systems. [3] However, simultaneous supplementation with vitamin C and E exhibited protective effect on sperm by inhibiting PCB induced reactive oxygen species (ROS) generation. [4] Administration of lycopene and ellagic acid showed similar protective effect. [5] These studies suggested that antioxidant nutrients can down regulate cytotoxic mechanisms of specific environmental contaminants.

However, conventional antioxidants neutralize ROS on a one to one basis, while PCB induced superoxide is sustained, so conventional antioxidants would

only be able to provide short term relief of oxidative stress. Danshen is one of the most versatile herbal drugs and has been used for hundreds of years to treat numerous disease. Lithospermate B (LAB) is an active component isolated from Danshen and found to have renoprotective properties due to its antioxidant effects. [6] And recent studies showed that LAB has protective effects on balloon injury induced neointimal hyperplasia and diabetic atherosclerosis. [7, 8]

Whether LAB could inhibit adverse effects of chemical toxicants, such as Aroclor 1254, to testis was unclear. This study was designed to evaluate the antioxidant effects of LAB on spermatogenesis after exposure to Aroclor 1254.

II. MATERIALS AND METHODS

1. Materials

Aroclor 1254 was purchased from Sigma Chemical Co, St. Louis, MO, USA. Other chemicals were of analytical grade and obtained from local commercial sources.

2. Animals and treatment

Eighty three days adult C57BL6 mice were purchased from Koatech (Pyeongtaek, Gyeonggi, Korea) and given free access to standard pellets and water during the experiments. After acclimatizing for 1 week (ninety days old), mice were divided into three groups. Each group consists of four animals. Group I: mice were intraperitoneally (i.p.) administered with corn oil and normal saline as vehicle for 30 days. Group II: mice were administered Aroclor 1254 at a dose of 2 mg/kg per day and normal saline i.p. for 30 days. Group III: mice were administered Aroclor 1254 at a dose of 2 mg/kg per day and LAB at a dose of 20 mg/kg per day i.p. for 30 days. The dosage and duration were selected as previous publications. The animals were fasted overnight, weighed and killed on the day following the last dosing. Testes and epididymides were quickly removed and cleared from the adhering tissues and then weighted.

3. Sperm count

The epididymis was placed in a petri dish containing 2 ml of phosphate buffered saline prewarmed to 37 ℃, and minced. After incubating at least 15 minutes at 37 ℃, a 0.5 ml sperm suspension was transferred to a test tube containing 2 ml phosphate buffered saline. The test tube was placed in a 60 ℃ water bath for 1 minutes and cooled to room temperature. Approximately 10 μl of diluted specimen was transferred to each of the counting chambers of a hemocytometer. After 2 minutes to allow the sperm to settle, the number of sperm was counted under 200× magnification.

4. Superoxide dismutase (SOD) Assay

The assay was performed with an SOD assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). Testes were homogenized with cold lysis buffer (20 mM HEPES, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose, pH 7.2) and centrifuged at 1,500 × g for 5 minutes at 4 ℃. The supernatant and the known amount (0, 0.025, 0.05, 0.1, 0.15, 0.2, 0.25 U/ml) of SOD (for standard) were mixed with tetrazolium salt and xanthine oxidase. After incubating at room temperature for 20 minutes, the absorbance at 450 nm was read.

5. Catalase (CAT) Assay

The assay was performed with a CAT assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). Testes were homogenized with cold lysis buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7.0) and centrifuged at 10,000 × g for 15 minutes at 4 ℃. The supernatant and the known amount (0, 5, 15, 30, 45, 60, 75 μM) of formaldehye (for standard) were mixed with methanol and hydrogen peroxide and incubated at room temperature for 20 minutes. To terminate the reaction, potassium hydroxide was added, and then, 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole was added. After incubating at room temperature for 10 minutes, potassium periodate was added, and incubated at room temperature for another 5 minutes. The absorbance at 540 nm was read.

6. Glutathione peroxidase (GPx) Assay

The assay was performed with a GPx assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). Testes were homogenized with cold lysis buffer (50 mM Tris-HCL, 5 mM EDTA, 1 mM DTT, pH 7.5) and centrifuged at 10,000 × g for 15 minutes at 4 ℃. The supernatant was mixed with co-substrate mixture (contain NADPH, glutathione, and glutathione reductase) and cumene hydrogen peroxide. The absorbance at 340 nm was read every minute for six minutes. The GPx activity was calculated with the specific formula.

7. Glutathione reductase (GR) Assay

The assay was performed with a GR assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). Testes were homogenized with cold lysis buffer (50 mM potassium phosphate, 1 mM EDTA pH 7.5) and centrifuged at 10,000 × g for 15 minutes at 4 ℃. The supernatant was mixed with oxidized glutathione and NADPH. The absorbance at 340 nm was read every minute for six minutes. The GR activity was calculated with the specific formula.

8. Hydrogen peroxide (H_2O_2) Assay

Generation of hydrogen peroxide was quantified with a QuantiChrom™ Peroxide Assay Kit (Bio-Assay Systems, Hayward, CA, USA). The kit is designed to measure peroxide concentration in biological samples without any pretreatment. The method utilizes the chromogenic Fe^{3+}-xylenol orange reaction, in which a purple complex is formed when Fe^{2+} provided in the reagent is oxidized to Fe^{3+} by peroxides present in the sample. The intensity of the color, measured at 585 nm, is an accurate measure of peroxide level in the sample. A standard curve was generated as recommended by the manufacturer using known amounts of H_2O_2 (conversions: 1 μM H_2O_2 equals 34 ng/ml).

9. Statistical analysis

Data are shown as the mean ± standard error of the mean. Comparisons of body weights, weight of testis and epididymis, sperm density, SOD, CAT, GPx,

GR and H_2O_2 among control and experimental groups were determined by one-way analysis of variable (ANOVA). Statistical significance was considered at the level of P value less than 0.05. All data were analyzed using PASW Statistics version 18 (SPSS Inc., Chicago, IL, USA).

III. RESULTS

1. Body weight, weight of testis and epididymis

The mean values of body weight, absolute and relative weight of testis and epididymis at the end of study are presented in Table 1. We observed no statistically significant differences in body weight, absolute and relative weight of testis and epididymis among 3 groups.

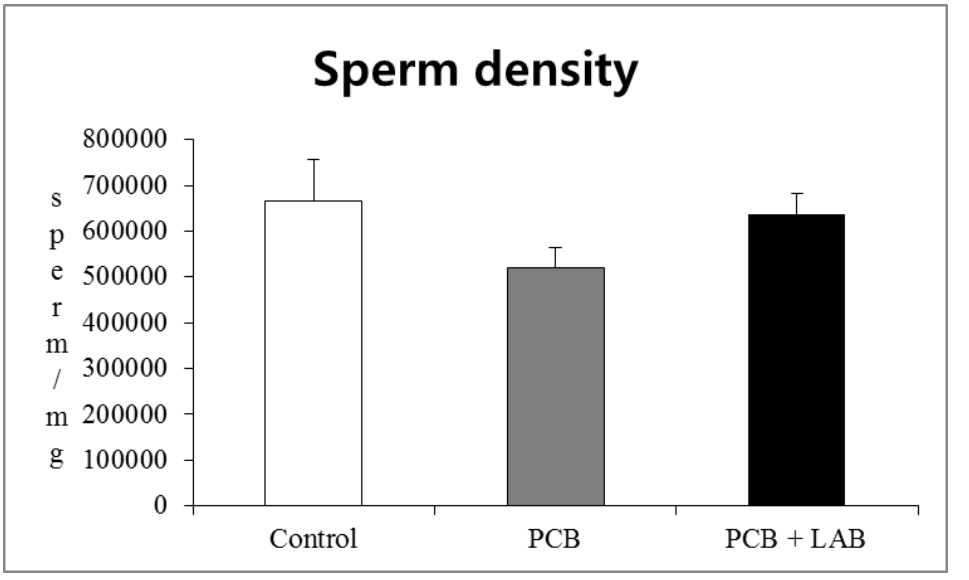
Table 1. Body weight, absolute and relative weight of testis and epididymis of control and experimental animals. Each value represents mean ± SEM of four animals. Significance at $P < 0.05$

Parameters	Control	PCB	PCB + LAB
Body weight (g)	31.1 ± 2.9	28.6 ± 0.8	28.4 ± 0.7
Absolute weight of testis (mg)	89.01 ± 8.72	102.74 ± 2.59	102.97 ± 4.67
Relative weight of testis (%)	0.29 ± 0.03	0.36 ± 0.01	0.36 ± 0.01
Absolute weight of epididymis (mg)	35.08 ± 2.23	35.46 ± 1.38	35.75 ± 1.00
Relative weight of epididymis (%)	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01

2. Sperm density

Sperm density was calculated by dividing the sperm count by the weight of epididymis. Figure 1 illustrates the sperm density in control and experimental groups. There were no statistically significant differences, but, sperm density was decreased in PCB alone treated group when compared to control group. However, PCB and LAB treated group showed a trend to increase in sperm density when compared to PCB alone treated group. Supplementation of LAB seemed to restore sperm density levels normal.

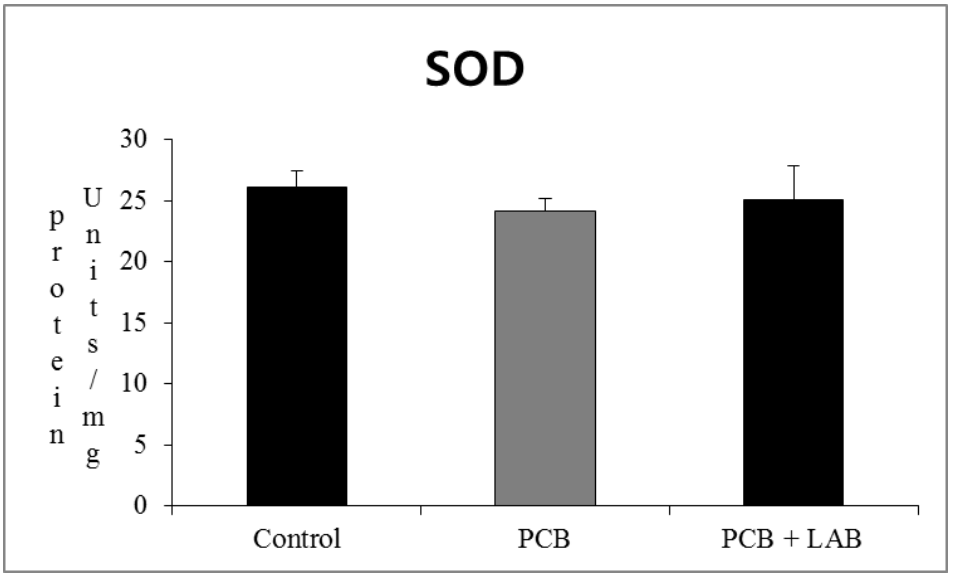
Figure 1. Effect of PCB and simultaneous administration of LAB on sperm density. Each bar denotes mean ± SEM of four animals. Significance at $P < 0.05$.

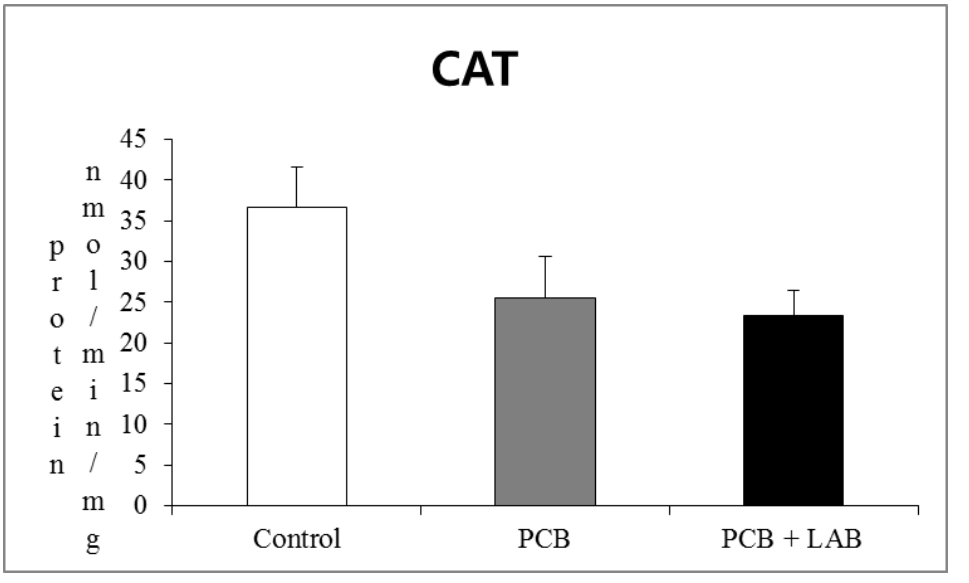


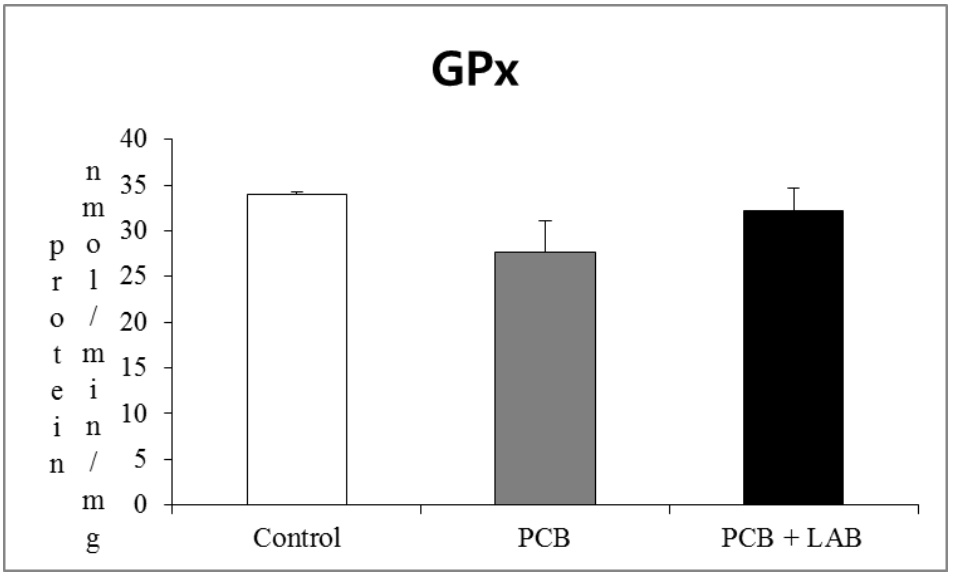
3. Activities of antioxidant enzymes

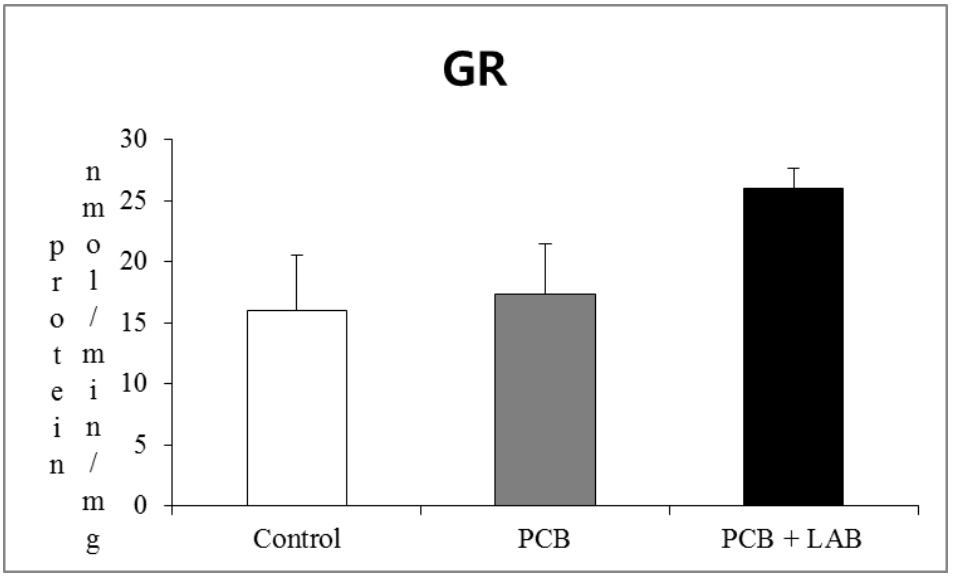
The activities of antioxidant enzymes such as SOD, CAT, GPx, GR in control and experimental animals are presented in figure 2. PCB alone treated group showed a trend to low in SOD, CAT, GPx when compared to control group. And PCB and LAB treated group showed a trend to high in SOD, GPx, GR when compared to PCB alone treated group. It seemed that supplementation of LAB restored antioxidant enzyme levels normal. But there were no statistically significant differences. Unfortunately, CAT in PCB and LAB group and GR in control group showed opposite trends.

Figure 2. Effect of PCB and simultaneous administration of LAB on SOD, CAT, GPx, GR. Each bar denotes mean ± SEM of four animals. Significance at $P < 0.05$.





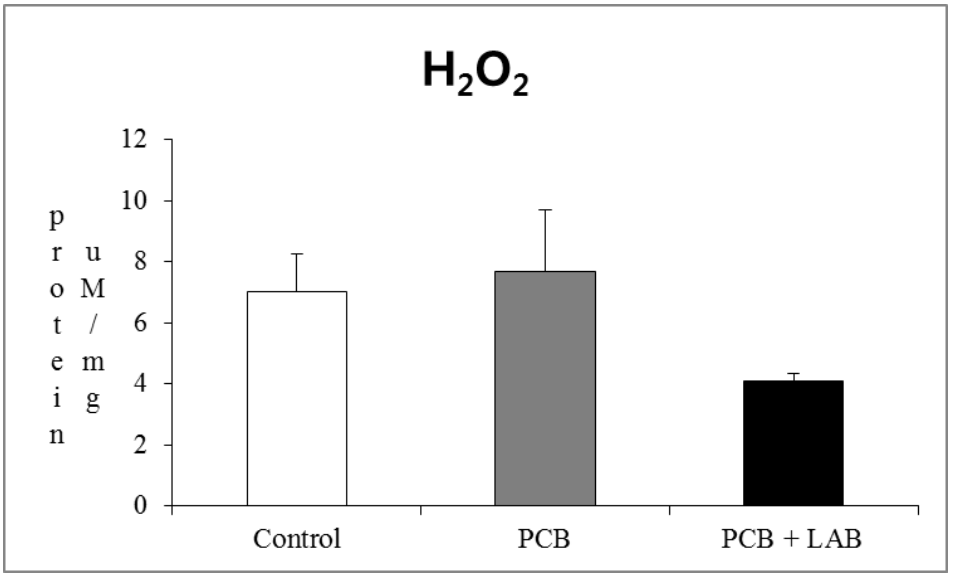

GPx
nmol/min/mg protein
40
35
30
25
20
15
10
5
0
Control
PCB
PCB + LAB


GR
nmol/min/mg protein
30
25
20
15
10
5
0
Control
PCB
PCB + LAB

4. Levels of H_2O_2

The levels of testis H_2O_2 are presented in figure 3. The data depict that PCB alone treated group showed a trend to high H_2O_2 levels when compared to control group. PCB and LAB treated group showed a trend to low H_2O_2 levels when compared to other groups. But there were no statistically significant differences.

Figure 3. Effect of PCB and simultaneous administration of LAB on H_2O_2. Each bar denotes mean ± SEM of four animals. Significance at $P < 0.05$.



IV. DISCUSSION

PCB is an environmental contaminant which is classified by World Health Organization as moderately hazardous. Effect of PCB on the male reproductive system has been studied by many researchers. The findings of the previous studies showed that PCB exposure reduced body weight and the weight of male reproductive organs. [3, 9-11] It might be due to the decreased bioavailability and production of androgens due to increased level of ROS. And some of them showed that antioxidant like vitamin C, E and lycopene supplementation brings back the body weight to normal due to its antioxidant effect. [9, 10] LAB is also known to have antioxidant effect. But, in this study, there were no statistically significant differences. There were also some studies alleged that PCB has no effect on these parameters. [12]

We found that the sperm density was slightly decreased in mice exposed to PCB. Decreased sperm density after exposure to PCB observed in this study may be explained by increased ROS levels in testicular tissues. And administration of LAB to PCB exposed mice showed a trend to prevent changes in the sperm density. It can be also explained by reducing production of ROS. Other studies showed that PCB can reduce sperm count and motility by decreasing Sertoli cell number, reducing fructose content in seminal vesicle and alternating steroid hormone metabolism. However, these cytostructural modifications and biochemical changes can be prevented by antioxidant and restore the sperm count and motility. [4, 5]

Oxidative stress occurs when the production of ROS exceeds the body's natural antioxidant defense systems, causing damage to macromolecules such as DNA, proteins and lipids. [13] To prevent the destructive effect of ROS, cells are provided with extensive antioxidant defense systems. These consist mainly antioxidant enzymes such as SOD, CAT, GPx, GR. ROS may be generated during the oxidative metabolism of PCB. SOD plays a key role in the detoxification of superoxide radicals. CAT and GPx are known to be responsible for the detoxification of H_2O_2. The activity of GPx is highly dependent on GSH

concentration. GSSG can be converted back to GSH by GR. Earlier reports have demonstrated that not only increased ROS and decreased antioxidant enzymes are involved in PCB induced testicular injury, but also antioxidants such as vitamin C, E, and lycopene have a protective role on PCB induced oxidative stress in testicular tissues. [9, 10, 14] In this study, PCB treatment has a trend to increase H_2O_2 and decrease the specific activities of antioxidant enzymes such as SOD, CAT, GPx. However, administration of LAB caused decrease in H_2O_2 levels, and increase in SOD, GPx, GR activities (Unfortunately not CAT) when compared to PCB exposed mice. These results agree with previous reports, which demonstrated that LAB restores SOD and normalizes CAT. [15] Therefore, the protective effect of LAB in PCB induced testicular injury may be largely due to its antioxidant effects.

These improvements in testicular tissues, sperm density and oxidant/antioxidant balance after LAB administrations may be explained with their free radical scavenging and antioxidant capacity. Previous studies with LAB have suggested that their role in the scavenging ROS is a key in the protection against vascular diseases. [6-8] However, its protective effects under impaired spermatogenesis by oxidative stress have not been investigated until now. In this study, we showed that LAB has protective effect by enhancing of antioxidant enzyme activities against testicular injury induced by PCB

This study has several limitations, which should be complemented by further study. First, the sample size was too small to show a statistically significant difference. But our data showed similar trends to previous studies. Second, PCB and LAB were administered by intraperitoneal injection, not by oral route. But in previous other studies, there were no significant difference of effect between intraperitoneal injection and oral route. And last, the histopathological evaluation of the structure of a testis was not performed in this study. Therefore, we cannot comment about pathologic changes such as degeneration, desquamation, disorganization and reduction in germinal cells, intestinal edema and congestion. We can only assume from other studies. [5]

From this study, we can conclude that the excessive oxidative stress may reduce spermatogenesis in PCB exposed mice. In addition, LAB has protective

effects against testicular injury induced by PCB. These protective effects of LAB seemed to be closely involved with the enhancing of antioxidant enzyme activities. Therefore, antioxidants from food consumed by human beings and animals, such as LAB can attenuate the negative effects of environmental pollutants. And especially LAB may be used to prevent testicular injury induced by PCB and to treat male infertility as an antioxidant. Further studies will be required to determine the molecular mechanisms involved in inhibition of intracellular ROS by LAB.

V. CONCLUSION

In conclusion, the extensive oxidative stress induced by PCB may reduce spermatogenesis, and LAB has protective effect by enhancing of antioxidant enzyme activities against testicular injury induced by PCB. These findings suggest that LAB may provide clinical treatment for male infertility.

REFERENCES

1. Byrne JJ, Carbone JP, Hanson EA. Hypothyroidism and abnormalities in the kinetics of thyroid hormone metabolism in rats treated chronically with polychlorinated biphenyl and polybrominated biphenyl. Endocrinology 1987;121:520-7.
2. Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jr., et al. Male reproductive health and environmental xenoestrogens. Environ Health Perspect 1996;104 Suppl 4:741-803.
3. Murugesan P, Senthilkumar J, Balasubramanian K, Aruldhas MM, Arunakaran J. Impact of polychlorinated biphenyl Aroclor 1254 on testicular antioxidant system in adult rats. Hum Exp Toxicol 2005;24:61-6.
4. Krishnamoorthy G, Venkataraman P, Arunkumar A, Vignesh RC, Aruldhas MM, Arunakaran J. Ameliorative effect of vitamins (alpha-tocopherol and ascorbic acid) on PCB (Aroclor 1254) induced oxidative stress in rat epididymal sperm. Reprod Toxicol 2007;23:239-45.
5. Atessahin A, Turk G, Yilmaz S, Sonmez M, Sakin F, Ceribasi AO. Modulatory effects of lycopene and ellagic acid on reproductive dysfunction induced by polychlorinated biphenyl (Aroclor 1254) in male rats. Basic Clin Pharmacol Toxicol 2010;106:479-89.
6. Lee GT, Ha H, Jung M, Li H, Hong SW, Cha BS, et al. Delayed treatment with lithospermate B attenuates experimental diabetic renal injury. J Am Soc Nephrol 2003;14:709-20.
7. Hur KY, Seo HJ, Kang ES, Kim SH, Song S, Kim EH, et al. Therapeutic effect of magnesium lithospermate B on neointimal formation after balloon-induced vascular injury. Eur J Pharmacol 2008;586:226-33.
8. Hur KY, Kim SH, Choi MA, Williams DR, Lee YH, Kang SW, et al. Protective effects of magnesium lithospermate B against diabetic atherosclerosis via Nrf2-ARE-NQO1 transcriptional pathway. Atherosclerosis 2010;211:69-76.
9. Senthil kumar J, Banudevi S, Sharmila M, Murugesan P, Srinivasan N, Balasubramanian K, et al. Effects of Vitamin C and E on PCB (Aroclor 1254) induced oxidative stress, androgen binding protein and lactate in rat Sertoli cells. Reproductive toxicology 2004;19:201-8.
10. Elumalai P, Krishnamoorthy G, Selvakumar K, Arunkumar R, Venkataraman P, Arunakaran J. Studies on the protective role of lycopene against polychlorinated biphenyls (Aroclor 1254)-induced changes in StAR protein and cytochrome P450 scc enzyme expression on Leydig cells of adult rats. Reprod Toxicol 2009;27:41-5.
11. Aly HA, Domenech O, Abdel-Naim AB. Aroclor 1254 impairs

spermatogenesis and induces oxidative stress in rat testicular mitochondria. Food Chem Toxicol 2009;47:1733-8.
12. Hsu PC, Li MH, Guo YL. Postnatal exposure of 2,2',3,3',4,6'-hexachlorobiphenyl and 2,2',3,4',5',6-hexachlorobiphenyl on sperm function and hormone levels in adult rats. Toxicology 2003;187:117-26.
13. Bartsch H, Nair J. Ultrasensitive and specific detection methods for exocylic DNA adducts: markers for lipid peroxidation and oxidative stress. Toxicology 2000;153:105-14.
14. Murugesan P, Muthusamy T, Balasubramanian K, Arunakaran J. Studies on the protective role of vitamin C and E against polychlorinated biphenyl (Aroclor 1254)--induced oxidative damage in Leydig cells. Free Radic Res 2005;39:1259-72.
15. Yokozawa T, Dong E, Oura H, Kashiwagi H, Nonaka G, Nishioka I. Magnesium lithospermate B suppresses the increase of active oxygen in rats after subtotal nephrectomy. Nephron 1997;75:88-93.

ABSTRACT(IN KOREAN)

Aroclor 1254에 노출된 C57BL6 쥐의 정자형성에 Lithospermate B가 미치는 영향

<지도교수 이은직>

연세대학교 대학원 의학과

김 다 함

배경: Polychlorinated biphenyls (PCBs)은 생식기관에 산화 스트레스를 일으키는 것으로 알려져 있으며 몇몇 항산화제는 이것의 보호효과를 보여주었다. Lithospermate B (LAB)는 몇몇 이전 연구에서 항산화 효과를 보여주었다. 우리는 LAB이 PCB에 의해 유발된 고환손상에 비슷한 보호효과를 보이는지 실험하였다.

방법: 어른 C57BL6쥐를 세 군으로 나누었다. 첫번째 군은 옥수수기름과 생리식염수를 30일 동안 투여 받았다. 두번째 군은 하루 2 mg/kg의 Aroclor 1254와 생리식염수를 30일 동안 투여 받았다. 세번째 군은 하루 2 mg/kg의 Aroclor 1254와 하루 20 mg/kg의 LAB을 30일 동안 투여 받았다. 마지막 투여 24시간 후, 고환과 부고환을 제거하였다. 부고환에서 정자를 수집해 수를 계산하였다. 고환을 homogenize한 다음 항산화 효소의 활성도 (SOD, CAT, GPx, GR)와 ROS의 수준 (H_2O_2)을 측정하였다.

결과: 비록 통계학적으로 큰 차이는 없었지만 이 실험의 결과는

PCB가 정자 농도, 항산화 효소의 활성도는 감소시키고 ROS의 수준은 증가시키는 것을 보여준다. LAB의 보충은 이런 변수들을 정상화 시켜주는 것으로 보인다.

결론: 결론적으로, LAB은 항산화 효소의 활성도를 증가시킴으로 PCB에 의한 고환손상에 대응하여 보호효과를 보인다.

핵심되는 말 : lithospermate B, 정자형성, aroclor 1254