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Effect of caffeine and ethanol intake on di (2-ethylhexyl) phthalate (DEHP)-induced testicular atrophy

Shigeru Suna [1,*] and Fumihiko Jitsunari [2]

[1] *Private Health Research Laboratory, 14-22 Shinkita-machi, Takamatsu-shi, Kagawa 760-0001, Japan.*
[2] *Emeritus, Kagawa University, Japan.*

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Abstract

Background: Di(2-ethylhexyl) phthalate (DEHP) is the most widely used polyvinyl chloride (PVC) plasticizer. Therefore, DEHP pollution is spreading all over the world. In recent years, it has attracted attention as an endocrine disrupting chemical. In animal experiments using rodents, testicular toxicity has been confirmed by feeding diets containing DEHP. Mono(2-ethylhexyl) phthalate (MEHP), a potent oxidative stressor, is thought to be directly involved in testicular toxicity. On the other hand, caffeine and ethanol, which are hydroxyl radical scavengers, are taken in relatively large amounts from food and drink on a daily basis. Although the reproductive toxicity of DEHP in humans remains unclear, it is important to clarify the relationship between DEHP toxicity and caffeine and ethanol intake through animal experiments.

Method: To investigate the effects of caffeine and ethanol intake on DEHP-induced testicular atrophy, rats were fed a 1 w/w % DEHP diet and caffeinated water (0.05 w/w %) or ethanol water (2.5 or 5 v / v %) or ethanol plus caffeine water for one week.

Result: By administering 1 w/w % DEHP-containing diet for one week, significant testicular weight decrease noted in all cases, and testicular MEHP concentration in the control group and DEHP–only administered group showed a strong negative correlation with the relative testicular weight (as a percentage of body weight). This indicates that there is a close relationship between the tissue MEHP concentration and testicular atrophy in the target organ, the testis.

In addition, it was found that the increase in Fe, Cu, and Ca concentrations in the testis was closely related to the decrease in relative testicular weight. Furthermore, multiple linear regression analysis suggested that the MEHP concentration in the testis was significantly related to the increase in the Cu concentration in the testis. This suggests that MEHP oxidative stress may induce the SOD enzyme. Also, caffeine administration was found to slightly but significantly improve Zn levels.

On the other hand, caffeine treatment significantly inhibited the decrease in testicular weight. Also, the testicular weight decrease in the ethanol and caffeine administration groups was less than that in the DEHP-only administered group. These findings suggest that ethanol and caffeine may inhibit DEHP testicular atrophy. In addition, in this administration experiment, the relative testicular weight vs. testicular MEHP plot in the ethanol and caffeine administered groups was clearly distributed above the regression line obtained from the relationship between the relative testicular weight and testicular MEHP concentration in the control and DEHP-only administered groups. From this, it was suggested that administration of DEHP in combination with ethanol or caffeine reduced DEHP testicular toxicity. Furthermore, multiple linear regression analysis suggested that combined use of ethanol and caffeine had a combined effect of reducing testicular atrophy.

* Corresponding author: Shigeru Suna

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Conclusion: Daily use levels of caffeine and ethanol were found to reduce DEHP-induced testicular toxicity in rats

Keywords: DEHP; Testicular toxicity; Caffeine; Ethanol

1. Introduction

Di(2-ethylhexyl) phthalate (DEHP) is the most widely used polyvinyl chloride (PVC) plasticizer. PVC products are used in various fields such as building materials, water pipes, daily necessities, toys, medical equipment, blood transfusion tubes and blood transfusion bags. Among these PVC products, DEHP is contained in several percent to several tens of percent. Therefore, it is one of the chemical substances that people from infants to the elderly have many opportunities to come into contact with on a daily basis. In recent years, it has attracted attention as an endocrine disrupting chemical [1, 2]. In animal experiments using rodents, testicular toxicity has been confirmed by administering diets containing DEHP [3-5]. Furthermore, it has recently been shown that administration of low concentrations affects Sertoli cell function [6]. A metabolite of DEHP, mono(2-ethylhexyl) phthalate (MEHP), is thought to be directly involved in testicular toxicity. MEHP is a strong oxidative stressor. The mechanism of germ cell apoptosis induction is being elucidated [7-10]).These facts raise concerns about the effects of low levels of DEHP contamination on the human body. Although the toxicity of DEHP to humans remains unknown, several epidemiological studies [11-18] suggest an association between phthalate exposure and semen quality.

On the other hand, caffeine and ethanol, which are hydroxyl radical scavengers [19-23], are ingested in relatively large amounts through food and drink on a daily basis. Therefore, it is important to clarify the relationship between DEHP toxicity and caffeine and ethanol intake. This report shows the results of administering a DEHP diet to rats with caffeine and ethanol at concentrations similar to those found in humans on a daily basis, such as coffee and beer [24, 25].

2. Material and methods

2.1. Chemicals and Animal Diet

DEHP, caffeine and ethanol was purchased from Wako pure chemical industries Ltd. (Osaka, Japan). The chemical purities of DEHP, caffeine and ethanol were found to be >97%, >98% and >99.7%, respectively. CE-2 diets (Clea, Tokyo, Japan) containing DEHP by 1 w/w% were prepared by Oriental Yeast Company (Chiba, Japan). MEHP was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All other chemicals were the highest grade from commercial sources.

2.2. Animals and Ethics

Male Sprague-Dawley rats aged three-week-old purchased from Charles River (Kanagawa, Japan) were housed at the Laboratory Animal Center of Kagawa University. They were acclimated at 22–24 °C and 50–60% relative humidity with a 12-h light/dark cycle. The experiment protocols had the approval by the Kagawa University Animal Committee.

2.3. Experimental Design

In the first experiment, 4-week-old rats weighing 100-130 g were divided into control and treatment groups of at least 6 animals. Treatment groups received a DEHP (1 w/w %) diet and tap water, or a DEHP diet and caffeinated water (0.05 w/w %), or a control diet and caffeinated water for one week. At the end of the experiment, rats were sacrificed by ether anesthesia. The organs were removed and weighed. Cardiac blood Samples were collected in heparinized tubes and plasma was separated from whole blood by centrifugation at 1500 g. Organ and plasma samples were frozen at −40 °C until MEHP and metal analysis.

In a second experiment, rats weighing 100-130 g were divided into control and treatment groups of at least 6 animals. The treatment group was given DEHP diet and ethanol-diluted water (ethanol; 2.5 or 5 v/v %) or caffeine-ethanol mixed water (caffeine; 0.05 w/w % and ethanol; 2.5 or 5 v/v %) for one week.

At the end of the experiment, rats were sacrificed by ether anesthesia. The organs were removed and weighed. Cardiac blood Samples were collected in heparinized tubes and plasma was separated from whole blood by centrifugation at 1500 g. Organ and plasma samples were frozen at −40 °C until MEHP analysis.

2.4. Plasma and Testicular MEHP Analysis

The MEHP levels in organs and plasma were determined by high performance liquid chromatography [26].

2.5. Testicular Metal Analysis

A sample testis was weighed into a PTFE decontaminated decomposition vessel and 0.5 ml of acid mixtures of HNO_3/ $HClO_4$/ H_2SO_4 (50:50:1, v/v/v) was added. The vessel on a heating plate was held at 140 °C for 4 h, and heated to 200 °C until almost dry. The residue was dissolved in 2 ml of 0.1 M HCl and diluted appropriately. The concentrations of Ca, Cu, Fe and Zn were determined by "one-drop" flame atomic absorption spectrometry [27]. The analytical instrument was a Seiko-SAS 7500 model equipped with deuterium background correction (Seiko, Tokyo, Japan).

2.6. Statistical Analysis

Results were expressed as means ± standard deviations (SD). A one-way ANOVA test followed by Tukey's multiple comparison test and multiple linear regression analysis was performed to compare treatment groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. First experiment

The dose calculated from food and drinking water intake during the administration period was 1.1 g/kg/day in the DEHP-administered group and 0.09 g/kg/day in the caffeine-administered group.

Table 1 compares the DEHP and caffeine-administered groups. DEHP-administered groups showed a significant decrease in testicular weight compared to controls, but caffeine treatment significantly inhibited the decrease in testicular weight. As shown in Table 2, there were no significant differences in plasma and testicular MEHP levels in the DEHP-administered groups.

A significant negative correlation between relative testicular weight (as a percentage of body weight) and testicular MEHP concentration was found among rats treated with DEHP-free diet group (Control) and DEHP diet-only group. Most of the data plots for the DEHP + caffeine-administered group were scattered above the regression line (Figure 1).

Table 3 shows the metal concentrations in testis. Significant increases in testicular Fe, Cu, and Ca levels were noted in the DEHP diet-only group compared to controls. As shown in Figure 2, testicular Fe, Cu and Ca were closely correlated with relative testicular weight, but Zn was not. A stepwise multiple regression analysis using testicular metal concentration as the objective variable and relative testicular weight, testicular MEHP concentration, and caffeine treatment as explanatory variables showed that relative testicular weight and testicular MEHP were significant independent variables of testicular Cu concentration (Table 4). Only relative testicular weight had a significant effect ($p<0.001$) on Fe and Ca concentrations. In addition, caffeine treatment was found to significantly increase Zn levels ($p<0.05$).

Table 1 Body and testicular weights of rats treated with DEHP and caffeine for one week

Group	Initial body weight (g)		Final body weight (g)		Testes (g)			Relative testicular weight (%)		
	mean	SD	mean	SD	mean	SD		mean	SD	
Control (n=12)	113.3	11.4	173.6	13.4	1.60	0.19	###	0.92	0.09	###
Caffeine (n=6)	111.3	12.1	180.2	19.2	1.62	0.25	###	0.89	0.05	###
DEHP (n=12)	111.6	12.8	167.1	16.6	1.05	0.16	***	0.63	0.10	***
DEHP + Caffeine (n=12)	110.3	12.2	170.0	18.9	1.28	0.32	**, #	0.75	0.16	**, #

* P<0.05, ** P<0.01, *** P<0.001 : Significant differences as compared with control group.; # P<0.05, ## P<0.01, ### P<0.001 : Significant differences as compared with DEHP group.

Table 2 Plasma and testicular MEHP concentrations of rats treated with DEHP and caffeine for one week

Group	Plasma MEHP (μg/ml)		Testicular MEHP (μg/g)	
	mean	SD	mean	SD
Control (n=12)	BDL		BDL	
Caffeine (n=6)	BDL		BDL	
DEHP (n=12)	26.4	18.5	4.9	1.3
DEHP+Caffeine (n=12)	22.7	12.8	4.9	1.4

BDL: below the detection limit.

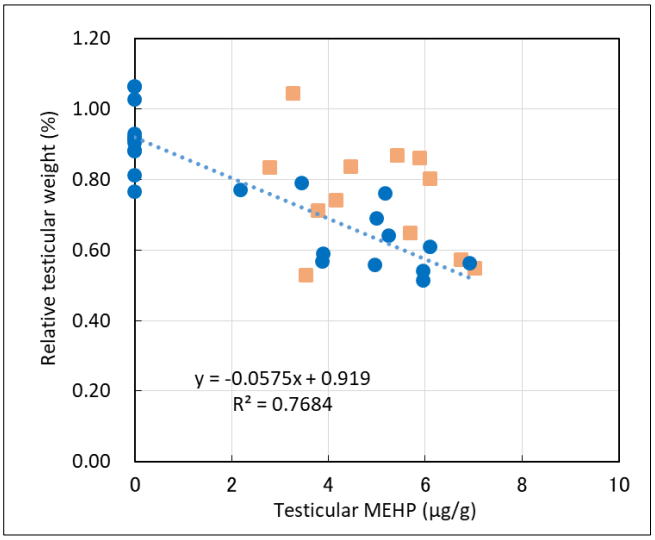

Figure 1 Relationship between relative testicular weight and testicular MEHP concentration in DEHP and caffeine treated groups. Filled circles; rats fed DEHP-free or DEHP diet and tap water. Filled squares; rats fed DEHP diet and caffeinated water

Table 3 Testicular metal concentrations of rats treated with DEHP and caffeine for one week

Group	Zn (ppm)		Fe (ppm)			Cu (ppm)			Ca (ppm)		
	mean	SD	mean	SD		mean	SD		mean	SD	
Control (n=6)	20.2	0.8	13.8	1.8		1.46	0.09		12.1	1.7	
Caffeine (n=6)	20.4	1.5	14.1	2.1		1.45	0.20		13.8	3.1	
DEHP (n=6)	17.8	2.4	20.9	2.2	**	2.24	0.23	**	17.3	3.7	*
DEHP + Caffeine (n=6)	22.0	2.7	17.8	4.9		2.07	0.60		15.0	3.1	

* P<0.05, ** P<0.01, *** P<0.001 : Significant differences as compared with control group.

3.2. Second experiment

The dose calculated from food and drinking water intake during the administration period was 0.9 g/kg/day in the DEHP-administered group, 1.5 -3.1 g/kg/day in the ethanol-administered group, and 0.09 g/kg/day in the caffeine-administered group.

Table 5 compares the DEHP, ethanol and ethanol plus caffeine-administered groups. The DEHP diet-only group showed a significant reduction in testicular weight compared to controls, whereas most of the ethanol- and caffeine-administered groups were non-significant. In addition, the 5% ethanol plus caffeine treatment group significantly suppressed the decrease in testicular weight.

As shown in Table 6, there were no significant differences in plasma and testicular MEHP levels in the DEHP-administered groups.

A significant negative correlation between relative testicular weight and testicular MEHP concentration was found among rats treated with DEHP-free diet group (Control) and DEHP diet-only group. Most of the data plots for the DEHP + ethanol or ethanol plus caffeine-administered group were scattered above the regression line (Figure 3, 4).

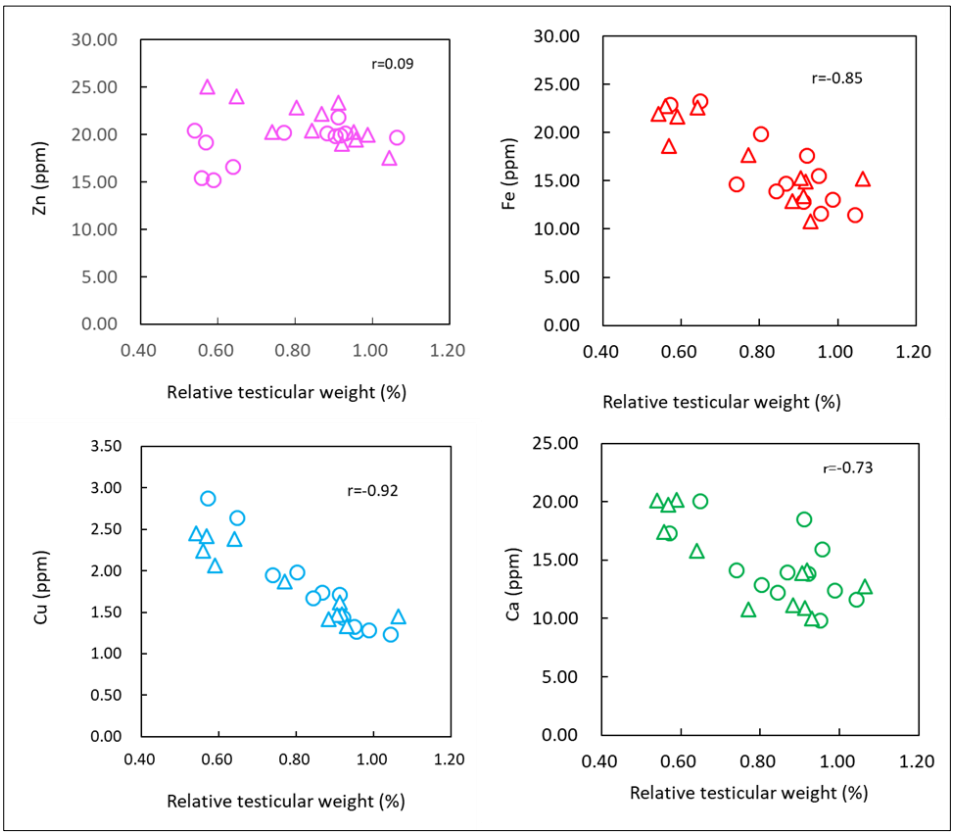

Figure 2 Relationship between testicular metal concentration and relative testicular weight in DEHP and caffeine treatment groups. Open circle; rats given the tap water. Open triangle; rats given the caffeinated water

Table 4 Stepwise multiple regression analysis with testicular Cu concentration as the objective variable, relative testicular weight, testicular MEHP concentration, and caffeine treatment as explanatory variables

Independent variables	Estimated regression oefficient B	95% confidence interval		Partial regression coefficient β	*P*-value	Cumulative R^2
		Lower bound	Upper bound			
Intercept	3.559	2.984	4.135	-	0.000	-
Relative testicular weight	-2269.000	-2.894	-1.645	-0.779	0.000	0.845
Testicular MEHP	0.035	0.000	0.069	0.215	0.050	0.872

Table 5 Body and testicular weights of rats treated with DEHP, ethanol and ethanol plus caffeine for one week

Group	Initial body weight (g)		Final body weight (g)			Testes (g)			Relative testicular weight (%)		
	mean	SD	mean	SD		mean	SD		mean	SD	
Control (n=6)	116.5	3.4	181.8	10.3	##	1.62	0.10	###	0.90	0.07	##
5% Ethanol (n=6)	123.0	2.6	180.7	4.5	##	1.68	0.15	###	0.93	0.07	###
DEHP (n=12)	118.3	2.9	167.1	4.8	**	1.00	0.20	***	0.60	0.12	***
DEHP+2.5% Ethanol (n=6)	118.1	3.7	175.8	8.5		1.25	0.32		0.71	0.15	
DEHP+5% Ethanol (n=6)	120.8	6.1	168.6	11.6		1.20	0.19		0.71	0.08	
DEHP+ 2.5% Ethanol /Caffeine (n=6)	117.0	5.8	169.7	8.0		1.13	0.20	*	0.67	0.13	
DEHP+ 5% Ethanol /Caffeine (n=12)	121.5	6.5	162.6	7.1	***	1.27	0.31		0.79	0.21	#

* P<0.05, ** P<0.01, *** P<0.001 : Significant differences as compared with control group.; # P<0.05, ## P<0.01, ### P<0.001 : Significant differences as compared with DEHP group.

Table 6 Plasma and testicular MEHP concentrations of rats treated with DEHP, ethanol and ethanol plus caffeine for one week

Group	Plasma MEHP (μg/ml)		Testicular MEHP (μg/g)	
	mean	SD	mean	SD
Control (n=6)	BDL		BDL	
5% Ethanol (n=6)	BDL		BDL	
DEHP (n=12)	52.4	13.5	6.8	2.1
DEHP+2.5% Ethanol (n=6)	49.9	21.7	8.3	3.8
DEHP+5% Ethanol (n=6)	53.5	10.5	6.8	1.8
DEHP+ 2.5% Ethanol/Caffeine (n=6)	41.0	7.9	8.9	2.4
DEHP+ 5% Ethanol/Caffeine (n=12)	56.7	16.8	6.5	3.3

BDL: below the detection limit

As shown in Table 7, Stepwise multiple linear regression analysis of using relative testicular weight as the objective variable, testicular MEHP, ethanol treatment and ethanol plus caffeine treatment as explanatory variables revealed that the ethanol and ethanol plus caffeine treatment group significantly inhibited testicular weight loss, and the inhibitory effect was greater in the ethanol plus caffeine treatment group.

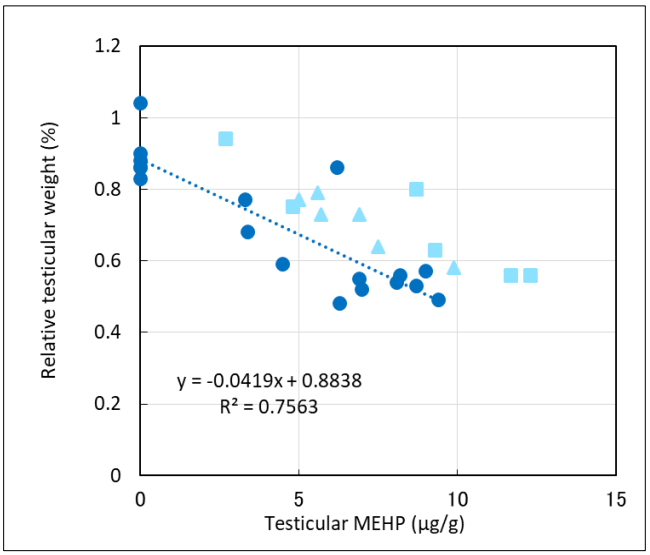

Figure 3 Relationship between relative testicular weight and testicular MEHP concentration in DEHP and ethanol treated groups. Filled circles; rats fed DEHP-free or DEHP diet and tap water. Filled squares; rats fed DEHP diet and 2.5% ethanol water. Filled triangles; rats fed DEHP diet and 5% ethanol water

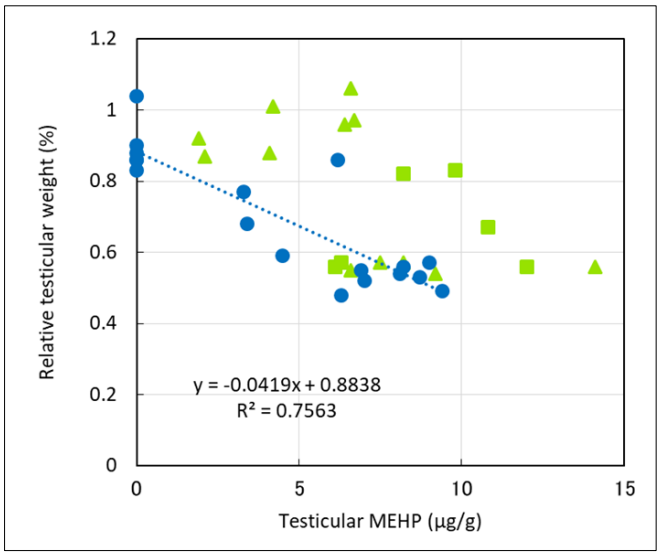

Figure 4 Relationship between relative testis weight and testicular MEHP concentration in DEHP and ethanol plus caffeine treated groups. Filled circles; rats fed DEHP-free or DEHP diet and tap water. Filled squares; rats fed DEHP diet + 2.5 % ethanol plus caffeine water. Filled triangles; rats fed a DEHP diet +5% ethanol plus caffeine water

Table 7 Stepwise multiple linear regression analysis with relative testicular weight as the objective variable and testicular MEHP concentration, ethanol treatment, and ethanol plus caffeine treatment as explanatory variables

Independent variables	**Estimated regression coefficient B**	**95% confidence interval**		**Partial regression coefficient β**	***P*-value**	**Cumulative R^2**
		Lower bound	**Upper bound**			
Intercept	0.856	0.789	0.923	-	0.000	-
Testicular MEHP	-0.035	-0.044	-0.027	-0.793	0.000	0.469
Ethanol/Caffeine	0.150	0.067	0.232	0.409	0.001	0.532
Ethanol	0.096	0.018	0.174	0.265	0.017	0.583

4. Discussion

Testicular atrophy is induced in rats by administration of a diet containing 1-2 w/w % DEHP [2-5]. Orally administered DEHP is hydrolyzed in the gastrointestinal tract of rats [28] and distributed mainly as MEHP [26]. In this study, we conducted two administration experiments using 4-week-old rats. In both experiments, by administering 1 w/w % DEHP-containing diet for 1 week, significant testicular weight decrease noted in all cases, and testicular MEHP concentration in the control group and DEHP–only administered group showed a strong negative correlation with the relative testicular weight. This indicates that there is a close relationship between the tissue MEHP concentration and testicular atrophy in the target organ, the testis.

In addition, in first experiment, it was found that the increase in Fe, Cu, and Ca concentrations in the testis was closely related to the decrease in relative testicular weight. Furthermore, multiple linear regression analysis suggested that the MEHP concentration in the testis was significantly related to the increase in the Cu concentration in the testis. This suggests that MEHP oxidative stress may induce the SOD enzyme [29, 30]. SOD contains a lot of Cu. Also, caffeine administration was found to slightly but significantly improve zinc levels, which may indicate that caffeine blocks germ cell apoptosis [31, 32].

On the other hand, caffeine treatment significantly inhibited the decrease in testicular weight in first experiment. Also in second experiment, the testicular weight decrease in the ethanol and caffeine administration groups was less than that in the DEHP-only administered group. These findings suggest that ethanol and caffeine may inhibit DEHP testicular atrophy. In addition, in this administration experiment, the relative testicular weight vs. testicular MEHP plot in the ethanol and caffeine administered groups was clearly distributed above the regression line obtained from the relationship between the relative testicular weight and testicular MEHP concentration in the control and DEHP-only administered groups. From this, it was suggested that administration of DEHP in combination with ethanol or caffeine reduced DEHP testicular toxicity. Furthermore, multiple linear regression analysis suggested that combined use of ethanol and caffeine had a combined effect of reducing testicular atrophy. Although the mechanism by which ethanol and caffeine inhibit DEHP testicular damage is unknown, there are some reports that low-concentration caffeine inhibits apoptosis in cultured cell systems [33, 34], suggesting that germ cell apoptosis is inhibited. In this administration experiment, ethanol was dissolved in drinking water at a concentration of 2.5-5 v / v % and caffeine was administered at a concentration of 0.05% (about 2.5 mM). These concentrations are almost the same as the concentrations of ethanol and caffeine in beverages that we consume on a daily basis, and it seems that ethanol and caffeine have no particular adverse effects on experimental animals

5. Conclusion

Daily use levels of caffeine and ethanol were found to reduce DEHP-induced testicular toxicity in rats.

Compliance with ethical standards

Acknowledgments

The author appreciates the help of colleagues in the Kagawa University.

Disclosure of conflict of interest

There is no conflict of interest in this work.

Statement of ethical approval

The experiment protocols had the approval by the Kagawa University Animal Committee.

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