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Effects of High Molecular Weight Water-Soluble Chitosan on *In Vitro* Fertilization and Ovulation in Mice Fed a High-Fat Diet

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A high molecular weight water-soluble chitosan (WSC) with an average molecular weight of 300 kD and a deacethylation level of over 90% was produced using a simple multi-step membrane separation process. It is known that WSC prevents obesity induced by a high-fat diet. Consequently, this study investigated whether or not WSC improved the ovarian dysfunction caused by obesity in mice. The mice were fed a high density protein and lipid diet for 4 weeks, followed by the administration of WSC at 480 mg/kg body weight per day for 4 days. Thereafter, the changes in body weight, ovulation rate, *in vivo* and *in vitro* fertilization and embryonic development were measured. WSC markedly reduced the body weight of obese mice fed with a high-fat diet, but not in mice fed with a normal diet. WSC had significant effects on the ovulation rate, both the *in vivo* and *in vitro* fertilization rates and embryonic development. These results indicate an improvement in the ovarian and oviduct dysfunction caused by obesity, and suggest an adjustment in the internal secretions and metabolic functions.

Key words: High molecular weight water-soluble chitosan (WSC), High-fat diet, Obesity, Infertility, Mouse

INTRODUCTION

Obesity is a metabolic problem that is caused by the ingestion of more fat than the body needs for physical activity and growth. It is characterized by an increase in the adipose tissue mass, adipocyte size and weight (Ross *et al.*, 1993; Hill *et al.*, 1992; Lemonnier, 1972; Obst *et al.*, 1981). This metabolic disease can be caused by a variety of causes such as hereditary factors, dietary habits, psychological impediments, central nervous system or hormonal disorders, and insufficient exercise. Obesity has important consequences for the reproductive system, depending upon the amount and distribution of body fat (Norman and Clark, 1998; Bray, 1997). Epidemiological evidence clearly shows that obesity contributes to menstrual disorders, reduced follicle numbers, polycystic ovary syndrome (PCOS), infertility, poor pregnancy outcome and impaired fetal well-being (Green *et al.*, 1988; Clark *et al.*, 1998; Grodstein *et al.*, 1994; Pasquali *et al.*, 1997). Obesity can also affect the ovulation rate and pregnancy (Clark *et al.*, 1995). As a result, obesity is one of major risk factors for a number of clinical diseases of the female reproductive system (Dechaud *et al.*, 1998).

Chitosan, a non-acetylated or partially deacetylated chitin (a linear homopolymer of -(1-4)-N-acetylglucosamine) has been proposed to be suitable biomaterial because of its apparent satisfactory biocompatibility (Kumar *et al.*, 1999; Shepherd *et al.*, 1997; Deuchi *et al.*, 1995). The mechanisms through which water-insoluble chitosan (chitosan solubilized in 1% acetic acid) can inhibit body weight increases induced by a high-fat diet are unclear. However, Han *et al.* previously reported that chitosan may function by reducing food absorption. Water-soluble chitosan has higher reactivity compared to water-insoluble chitosan (Kumar *et al.*, 1999). Recently, it had been reported that chitosan reduced the increased body weight and plasma triacylglycerol, cholesterol of

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obese mice (Han *et al.*, 1999). From this, the following hypothesis can be made: Obesity is one of the major risk factors for producing a variety of ovarian dysfunctions, which can be corrected, in part, by WSC through the reduction of body fat. Therefore, this study determined whether or not WSC could affect the in vitro fertilization and ovulation in mice fed a high-fat diet.

MATERIALS AND METHODS

Preparation of WSC

The WSC was dissolved in distilled water (32 mg/ml) and filtered through a 0.2 μm filter. The WSC solution at pH 6.0 was stored at 4°C.

Animals

The female B6C3F1 mice aged 4 weeks were supplied by the Korea Experimental Animals Center (Daejon, Korea), and were given access to a commercial diet *ad libitum* (Samyang, Korea) and tap water. The animals were housed under controlled conditions of 14 h light and 10 h darkness, at 23 ± 2°C and a relative humidity of 55-66%. The control mice were provided with laboratory pellet chow (Samyang, Korea; protein 24%, lipid 3.5%, carbohydrate 60.5%), and the high fat-diet group received a high fat-diet (beef tallow 40%, corn starch 10%, sugar 9%, vitamin mixture 1%, mineral mixture 4% and casein 36%) for 4 weeks. For the last 4 days of this period, the mice were treated with WSC orally at 480 mg/kg body weight per day, and all control animals received instead an equal volume of physiological saline. After WSC treatment for 4 days, the mice were treated with pregnant mare's serum gonadotropin (PMSG; Sigma, St Louis, USA) and human chorionic gonadotropin (hCG; Sigma) to induce multi-ovulation. The ratio of ovulated oocytes to normal oocytes was counted. The level of *in vitro* fertilization and embryonic development were examined.

Inducing superovulation

Superovulation was induced using a slight modification of the methods reported by Summer *et al.* (2000). Mice aged 8 weeks were intraperitoneally treated (0.01 ml/g body weight) with PMSG. 48 h later, 5 IU of hCG were intraperitoneally administered. The selected mice were sacrificed by a cervical dislocation 15 h after hCG administration.

Media

Table I shows the composition of the basic media, TYH and MWM, used for fertilizating the oocytes. The culture medium for early development of the mouse embryos was MWM. All fertilization and culture media (each 200 μl) were covered with paraffin oil (Fisher, USA) and equilibrated in an atmosphere of 5% CO_2 in air at 37°C overnight.

Table I. Composition of TYH and MWM media

Ingredient	TYH (mg/ml)	MWM (mg/ml)
NaCl	6.976	6.400
KCl	0.356	0.356
$CaCl_2$	0.190	-
KH_2PO_4	0.162	0.162
$MgSO_4 \cdot 7H_2O$	0.293	0.294
$NaHCO_3$	2.106	1.900
Calcium lactate, 5-hydrate	-	0.460
Glucose	1.000	1.000
Na-pyruvate	0.110	0.025
Penicillin G	0.075	0.075
Streptomycin	0.050	0.050
0.1% Phenol red	200 μl	100 μl
β-Mercaptoethanol (20 mM)	-	50 μl
EDTA·2Na (100 mM)	-	50 μl
BSA	4	3

Preparation of sperm suspension

A dense mass of spermatozoa was isolated from the epididymis of 9-week old B6C3F1 mice using standard procedures (Toyoda and Chang., 1974; Oh *et al.*, 1998), and placed in 200 μl of TYH, which had been covered with paraffin oil (Fisher, USA). They were then incubated at 37°C in a 5% CO_2 air atmosphere for 1 h. The sperm concentrations were determined by a haemocytometer.

Oocyte collection

Female mice were superovulated by an intraperitoneal injection of 5 IU PMSG, followed by an injection of 5 IU hCG 48 h later. The oocytes were collected from the oviducts between 13.5 and 14 h after hCG administration. The oviducts were isolated and placed in a dish containing paraffin oil. The cumulus-oocyte complexes were dissected from the swollen ampulla, and transferred to the TYH medium under paraffin oil, followed by preincubation at 37°C in a 5% CO_2 air atmosphere.

In vitro Fertilization

In vitro fertilization was carried out in drops of MWM under paraffin oil. A preincubated, capacitated sperm suspension was added gently to freshly ovulated oocytes to give a final motile sperm concentration of 1×10^6/ml. The combined spermoocyte suspension was incubated for 5 h. The oocytes were then washed through several changes

of medium and finally incubated in 40 μl drops of medium under paraffin oil. Fertilization was confirmed by recording the number of 2-cell embryos 24 h after completing the *in vitro* fertilization (summers *et al.*, 2000).

Micromanipulation

The fertilized embryos were incubated in TYH medium for 15-30 min prior to micromanipulation. The oocytes were microinjected in 50 μl drops mounted on microscopes (Carl Zeiss). The glass micropipettes (Sigma, USA) were filled by suction from a microdrop containing different concentrations of WSC. All regents were injected into the cytoplasm. The injection volume was approximately 5 to 10 μl. After manipulation, the oocytes were cultured in individual microdrops (about 50 μl) under light mineral oil.

Reagents

High molecular weight water-soluble chitosan (average molecular weight 300,000 Da) was obtained from Chito 153 Co., Ltd.

Statistical analysis

The values obtained in this study are expressed as a mean ± SEM. The statistical analyses used were the ANOVA and Duncan's multiple range test using the STATVIEW program (Abacus Concepts, Inc., Berkeley, CA). p values < 0.05 wereconsidered to be significant.

RESULTS

Changes of body weight

After treating the mice with or without WSC for 4 days, the temporal changes in body weight were observed (Table II). WSC did not significantly affect the body weights in normal mice, but did in the obese mice. The increased body weights of the obese mice were lowered significantly by administering WSC at 480 mg/kg body weight per day (p<0.05).

Table II. Changes of body weight

Group		WSC treatment		BW change (g)
		Before (g)	After (g)[a]	
Normal	Control[b]	21.19 ± 1.53	22.73 ± 0.81	+ 1.54
	WSC[c]	22.15 ± 1.28	22.94 ± 0.99	+ 0.79
HF	Control	27.19 ± 1.05	28.63 ± 0.92	+ 1.44
	WSC	27.56 ± 1.00	23.22 ± 0.44	- 4.34*

[a]At 8 weeks after the beginning of the experiment, body weight was measured. Results are expressed as mean ± S.E.
[b]Animals were orally treated with saline.
[c]Animals were orally treated with WSC at 480 mg/kg body weight per day for 4 days.
*P<0.05; significantly different from the control.
HF = high-fat diet; WSC = high molecular weight water-soluble chitosan; BW = body weight

Effects of WSC on ovulation rate

As shown in Table III, in the mice fed a normal diet, the number of ovulated oocytes increased after WSC treatment (33.7 ± 6.3 to 38.0 ± 0.7, p<0.05). Moreover, a similar result was also observed in the mice fed with a high-fat diet. The number of ovulated oocytes was higher in the WSC-treated mice (34.4 ± 3.4) compared to control mice (30.4 ± 4.2). WSC did not dramatically change the number of normal oocytes among the ovulated oocytes in mice fed with normal diet. In contrast, the number of normal oocytes among ovulated oocytes in WSC-treated mice (93.6%) was significantly higher in mice fed a high-fat diet than that in the control animals (84.4%, p<0.05).

In vivo fertilization and early embryonic development

After treating the mice with or without WSC for 4 days, *in vivo* fertilization and the rate of embryonic development to the blastocyst stage were examined (Table IV). In the mice fed a high-fat diet, the *in vivo* fertilization rates in WSC-treated mice were significantly higher compared those of the control mice, being 90.7% for the WSC-

Table III. Effects of WSC on ovulation

Group		No. of mice (%)[a]	No. of oocyte per mouse ovulated[b]	No. of normal oocyte per mouse ovulated[b]
Normal	Control[c]	12/15(80.0)	33.67 ± 6.25	31.83 ± 5.71
	WSC[d]	14/15(93.3)	38.00 ± 0.72	36.29 ± 3.42
HF	Control	14/15(93.3)	30.40 ± 4.22	25.67 ± 3.70
	WSC	14/15(93.3)	34.40 ± 3.36	32.20 ± 3.56*

[a]At 8 weeks after the beginning of the experiment, ovulation was measured.
[b]Data are expressed as mean ± S.E.
[c]Animals were orally treated with saline.
[d]Animals were orally treated with WSC at 480 mg/kg body weight per day for 4 days.
*P< 0.05; significantly different from the control.
HF = high-fat diet; WSC = high molecular weight water-soluble chitosan; BW = body weight

treated mice and 82.8% for the control mice. However, WSC did not affect the fertilization rates of mice fed a normal diet. In the mice fed a high-fat diet, embryonic development to the blastocyst stage was significantly higher in the WSC-treated mice (81.2%) compared to the control mice (48.9%), but was not significantly different in mice fed a normal diet WSC-treated 85.1%, control mice, 82.3%.

In vitro fertilization and early embryonic development

After treating the mice with or without WSC for 4 days, the *in vitro* fertilization and the rate of embryonic development to the blastocyst stage were examined (Table V). In the mice fed a high-fat diet, the in vitro fertilization rates in the WSC-treated mice were significantly higher compared those of the control mice, being 89.3% for the WSC-treated mice and 77.8% for the control mice. However, WSC did not affect the fertilization rates in mice fed a normal diet. In mice fed a high-fat diet, embryonic development to the blastocyst stage was significantly higher in the WSC-treated mice compared to the control mice, being 81.1% in the WSC-treated mice and 45.4% in the control, but were no significantly differenced in the mice fed a normal diet (WSC-treated mice, 80.7%; control mice, 84.8 %).

Microinjection of WSC into embryos

To estimate whether or not WSC would directly affect *in vitro* fertilization and embryonic development, WSC was microinjected at various concentrations (3, 30 and 300 ng/embryo) into the embryo cytoplasm. As shown in Table VI, WSC at 300 ng/embryo significantly increased embryonic development in embryos originating from the mice fed a high-fat diet, but not in the embryos isolated from mice fed a normal diet.

DISCUSSION

Chitosan can be obtained from biomass and is considered to be a polyamine $(-NH_2)_n$. However, its applica-tions are limited due to its insolubility in most solvents including water. Commercial chitosans with a degree of deacetylation (DA) below 70% consist of a mixture of various low

Table IV. *In vivo* fertilization and early embryonic Development

Group		No. of oocyte	Fertilized embryos (%)[a]	%[b] of embryos developing to			
				2 cell (24)[c]	4 cell (48)[c]	Morula (72)[c]	Blastocyst (96)[c]
Normal	Control[d]	32	87.2 ± 7.3	82.3 ± 8.2	82.3 ± 8.2	82.3 ± 8.2	82.3 ± 8.2
	WSC[e]	34	92.2 ± 4.8	85.1 ± 5.6	85.1 ± 5.6	85.1 ± 5.6	85.1 ± 5.6
HF	Control	30	82.8 ± 2.9	70.6 ± 4.7	61.1 ± 8.3	54.7 ± 9.1	48.9 ± 7.1
	WSC	31	90.7 ± 3.3*	85.2 ± 4.7	83.6 ± 3.6	82.7 ± 3.2	81.2 ± 3.8*

[a]Oocytes were examined at 5 h after insemination, and only embryos were cultured. Experiments were replicated 6 times.
[b]Data are expressed as mean ± S.E.
[c]Numbers in parentheses indicate the time of examination (hours after insemination).
[d] nimals were orally treated with saline water.
[e]Animals were orally treated with WSC at 480 mg/kg body weight per day for 4 days.
*P< 0.05; significantly different from the control.
HF = high-fat diet; WSC = high molecular weight water-soluble chitosan.

Table V. *In vitro* fertilization and early embryonic development

Group		No. of oocyte	Fertilized embryos (%)[a]	%[b] of embryos developing to			
				2 cell (24)[c]	4 cell (48)[c]	Morula (72)[c]	Blastocyst (96)[c]
Normal	Control[d]	34	90.0 ± 4.2	84.3 ± 5.9	82.1 ± 6.3	80.7 ± 6.5	80.7 ± 6.5
	WSC[e]	32	89.0 ± 6.0	84.8 ± 6.6	84.8 ± 6.6	84.8 ± 6.6	84.8 ± 6.6
HF	Control	30	77.8 ± 19.1	65.3 ± 15	57.8 ± 17.1	51.0 ± 15.1	45.4 ± 13.6
	WSC	31	89.3 ± 5.3*	85.2 ± 6.8	84.0 ± 6.0	81.1 ± 4.9	81.1 ± 4.9*

[a]Oocytes were examined at 5 h after insemination, and only zygotes were cultured. Experiments were replicated 6 times.
[b]Data are expressed as mean ± S.E.
[c]Numbers in parentheses indicate the time of examination (hours after insemination).
[d]Animals were orally treated with saline.
[e]Animals were orally treated with WSC at 480 mg/kg body weight per day for 4 days.
*P< 0.05; significantly different from the control.
HF = high-fat diet; WSC = high molecular weight water-soluble chitosan.

Table VI. Microinjection of WSC into embryos

Group		WSC (ng)	No. of Fertilized embryos[a]	%[b] of embryos developing to			
				2 cell (24)[c]	4 cell (48)[c]	Morula (72)[c]	Blastocyst (96)[c]
Normal	Control[d]	-	37	91.9 ± 4.2	91.9 ± 4.2	89.2 ± 5.3	89.2 ± 5.3
	WSC[e]	300	40	90.0 ± 6.8	87.5 ± 5.9	87.5 ± 5.9	87.5 ± 5.9
		30	35	88.6 ± 3.3	88.6 ± 3.3	85.7 ± 6.6	85.7 ± 6.6
		3	33	90.9 ± 4.8	87.8 ± 5.9	87.8 ± 5.9	87.8 ± 5.9
HF	Control	-	30	66.6 ± 8.3	53.3 ± 9.1	53.3 ± 9.1	53.3 ± 9.1
	WSC	300	35	94.3 ± 4.8	88.6 ± 5.6	88.6 ± 5.6	88.6 ± 5.6*
		30	30	66.6 ± 3.2	66.3 ± 4.3	66.3 ± 4.3	66.3 ± 4.3
		3	25	68.0 ± 4.7	64.0 ± 6.0	60.0 ± 4.9	60.0 ± 4.9

[a]Oocytes were examined at 5 h after insemination, and only zygotes were cultured. Experiments were replicated 6 times.
[b]Data are expressed as mean ± S.E.
[c]Numbers in parentheses indicate the time of examination (hours after insemination).
[d]Animals were orally treated with saline.
[e]Animals were orally treated with WSC at 480 mg/kg body weight per day for 4 days.
*$P<0.05$; significantly different from the control.
HF = high-fat diet; WSC = high molecular weight water-soluble chitosan.

molecular weight chitosans and are partially soluble in acid. Therefore, additional purification processes are required. WSC, which has an average molecular weight of 300,000 Da and a DA of over 90%, can be produced using a simple multi-step membrane separation process (Kim *et al.*, 1999). The initial chitosan has an ionic form (-NH_3^+) in the structure (Illum, 1998). Previously, chitosan was shown to prevent an increase in body fat caused by a high-fat diet through its ability to inhibit the intestinal absorption of dietary fat in obese mice (Han *et al.*, 1999; Muzzarelli, 1999). In the present study, the effects of WSC on fertilization and early stage of embryonic development in obese mice were examined. WSC markedly reduced the body weight of mice that was increased by the high-fat diet, and significantly increased the fertilization rates and early stage of embryonic development in obese mice. Therefore, these results suggest that ovarian dysfunctions caused by obesity may be improved by the oral administration of WSC.

Previous biochemical studies showed that obesity produces a variety of alterations in the reproductive system, depending upon the body fat distribution (Norman and Clark, 1998; Bray, 1997). A loss of body weight may restore the abnormal alterations of reproductive system. Here, we observed that the body weight that was increased by the high-fat diet was significantly decreased by WSC (Table II). These results suggest that the weight loss induced by WSC administration may lead to improvements in the ovulation and fertility rates indicating that obesity affects ovulation. This hypothesis could be also supported by a previous report (Clark *et al.*, 1995). In the present study, a decrease in the ovulation rate in mice fed a high-fat diet compared to mice fed a normal diet was compared, and this reduction was completely recovered by treating the obese mice with WSC. The results suggest that WSC may improve the ovulation rate reduced by obesity in mice fed a high-fat diet (Table III). In the past few years, obesity, particularly abdominal obesity, has been found to impair fecundity and to reduce the pregnancy rate during infertility treatment (Zaadstra *et al.*, 1993; Wass *et al.*, 1997). In addition, obesity is an independent risk factor for early pregnancy loss (Peter *et al.*, 2000). In terms of these suggestions, this study examined the effects of WSC on the fertilization rate and embryonic development, and showed that WSC conspicuously improved the rates of *in vivo* and *in vitro* fertilization and embryonic development in mice fed with high-fat diet (Tables IV and V). These results may be directly and/or indirectly related to the loss of body weight in obese mice.

In conclusion, this study illustrated the effects of WSC on ovarian function in mice fed a high-fat diet. WSC has significant effects on weight loss, the ovulation rates, the *in vivo* and *in vitro* fertilization rates and embryonic development in mice with obesity induced by the high-fat diet, but has no affect in normal mice. These findings suggest that WSC might improve the functions of the ovary and the oviduct in obese mice.

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