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Original Article

# Pengaruh Suplementasi Susu Kedelai Dan Genistein Sub Kronis Terhadap Infiltrasi Lemak Pankreas Tikus Jantan Sprague Dawley

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TENTANG ARTIKEL

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A B S T R A K

Isoflavon (genistein, daizein) pada susu kedelai bersifat sebagai fitoestrogen. Pada ras Asia kadar fitoestrogen dalam darah mencapai 160ng/ml, 80 kali lebih tinggi dari penduduk barat. Kadar fitoestrogen yang tinggi berpotensi mengganggu fungsi sistem endokrin karena dapat berikatan dengan reseptor estrogen. Contohnya yatiu pemberian genistein subkronis dapat menurunkan ekspresi reseptor estrogen dan androgen pada prostat sehingga menurunkan ukuran prostat. Fungsi dan distribusi jaringan adiposit diregulasi reseptor estrogen, penurunan ekspresi *Estrogen Receptors Alpha* (ERα) menyebabkan distribusi lemak ektopik pada jaringan viseral, antara lain infiltrasi lemak pada pankreas. Paparan subkronis susu kedelai dan genistein diperkirakan juga dapat mengganggu fungsi dan distribusi jaringan adiposit melalui aktivasi jalur *Estrogen Receptors Beta* (ERβ). **Tujuan :** Mengetahui pengaruh suplementasi susu kedelai dan genistein subkronis suplementasi terhadap infiltrasi lemak pankreas. **Metode**: Sebanyak 35 ekor tikus Sprague dawley jantan dibagi menjadi 7 kelompok perlakuan, yaitu kontrol negatif dengan pemberian pakan standar, suplementasi susu kedelai dosis rendah, sedang, dan tinggi (100mg, 200mg, dan 400mg), serta suplementasi genistein dosis rendah, sedang dan tinggi (0,4 mg, 0,8 mg, dan 1,6 mg). Pemberian perlakuan selama 60 hari. Pada akhir perlakuan dilakukan pemeriksaan histologi derajat infiltrasi lemak pankreas. Analisis komparasi dilakukan menggunakan uji Kruskal Wallis dilanjutkan uji post hoc dengan Mann Whitney. **Hasil:** Pada kelompok kontrol didapatkan infiltrasi lemak pada pankreas derajat sedang sampai berat, dan terdapat tren penurunan derajat infiltrasi lemak pankreas pada kelompok suplementasi susu kedelai dan genistein subkronis yang searah dengan peningkatan dosis dibanding kontrol. Namun secara statistik tidak didapatkan perbedaan bermakna derajat penurunan infiltrasi lemak pankreas pada suplementasi susu kedelai dan genistein subkronis dengan kelompok kontrol. **Kesimpulan:** Pemberian suplementasi susu kedelai dan genistein subkronis tidak bermakna menurunkan derajat infiltrasi lemak pankreas tikus jantan Sprague Dawley.
*Kata Kunci:* Susu Kedelai, Genistein, Infiltrasi Lemak Pankreas

# The Impact of Subchronic Soybean Milk and Genistein Supplementation on Pancreatic Fatty Infiltrations of Sprague Dawley Male Mice

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ABSTRACT

Isoflavones (genistein, daidzein) on soybean milk have phytoestrogenic properties. In Asian, the blood phytoestrogen levels can reach 160 ng/ml (80 times higher than Western). This may potentially disrupt endocrine functions regarding its binding with estrogen receptors.. Since the function and distribution of adipose tissues are regulated by estrogen receptors, the reduction of estrogen receptor-α(ERα) results in ectopic fats distribution around visceral tissues, such as the pancreas. **Aim:** To investigate the impact of subchronic soybean milk and genistein supplementation on pancreatic fatty infiltrations in mice. **Methods:** The experiment used 35 Sprague dawley male mice under 7 treatment groups within 60 days: negative control with standard rationed food, 3 groups with variable dose of soybean milk: 100 mg, 200 mg, and 400 mg, and 3 groups with variable dose of genistein: 0.4 mg, 0.8 mg, and 1.6 mg. Histological measurements on the level of pancreatic fatty infiltrations were conductedafter. Analyses used Kruskal-Wallis and post-hoc Mann-Whitney. **Results:** Medium to high level of pancreatic fatty infiltrations was found at the control group while there is a decreasing trend on the level of pancreatic fatty infiltrations on groups with soybean milk and subchronic genistein compared with control group, proportional to higher dosage supplementation. Reduction of pancreatic fatty infiltration levels on groups with soybean milk and subchronic genistein supplementation is not statistically significant compared to control. **Conclusion:** Supplementation of soybean milk and subchronic genistein do not significantly reduce the levels of pancreatic fatty infiltrations in Sprague dawley male mice.

*Keywords:* soybean milk, genistein, pancreatic fatty infiltrations

## INTRODUCTION

Soybean and its derivatives are the main source of vegetable protein for the Asian population.[1-3] Isoflavones found in soybeans (genistein, daizein, glysitein) have the benefit of increasing high-density lipoprotein (HDL) levels and reducing low-density lipoprotein (LDL) levels and have antioxidant effects.[4]

Isoflavones in soybeans are phyto-estrogens. In the population of Asia, the level of phytoestrogens in the blood reaches 160ng / ml (80 times higher than the western population).[5] Phytoestrogens can bind to estrogen receptors so that by WHO categorized as *Endocrine Disrupting Chemicals* (EDC) because they have the potential to interfere with endocrine function.[6] Previous studies have shown that in male experimental animals, subchronic administration of genistein can reduce the expression of estrogen and androgen receptors in the prostate, thereby reducing prostate size.[7]

The function and distribution of adipocyte cells are regulated by estrogen receptors.[8] Healthy adipose expansion in the form of adipocyte hyperplasia, subcutaneous fat distribution, and high adinopectin secretion is regulated by normal ERα expression. Meanwhile, pathological adipose expansion is in the form of adipocyte hypertrophy and fat infiltration to the visceral organs. Furthermore, there is a decrease in insulin sensitivity, a tendency to develop metabolic syndrome, and inflammation in pancreatic beta cells which increase the risk of *pancreatic malignancy*.[8-11]

The nature of soy milk as an EDC raises a debate about the benefits and ill effects of soy and its derivatives, especially in the long term.[12, 13] To date, no studies have specifically examined the soy milk and genistein supplementation effect on pancreatic fat infiltration in males. This study aims to determine the effect of supplementation of soy milk and sub chronic genistein on pancreatic fat infiltration of Sprague Dawley male mice.

## METHODS

### Study Design

This study used a true experimental in vivo test design. Post-test only done to control group.

### Experiment and Treated Animals

The study was conducted on *Sprague Dawley* male mice those were 6-8 weeks old and 160-250 grams body weight. The experimental protocol has been approved by the Research Ethics Commission of Faculty of Medicine, Universitas Brawijaya by number 96/EC/ KEPK/03/2017 and conducted at the Biomedical Laboratory of the Faculty of Medicine, Universitas Brawijaya. A total of 35 Sprague Dawley male mice, divided into 7 treatment groups. The negative control group was only given standard rationed food. The treatment group was supplemented with soy milk 100 mg, 200 mg, and 400 mg and pure genistein supplementation with 0.4 mg, 0.8 mg, and 1.6 mg. This dosage is a conversion of genistein consumption in humans, which is 20 mg, 40 mg, and 80 mg/day.[1] Duration of treatment for 60 days. Subjects who died were excluded from the study. Body weight, height, and *body mass index* (BMI) were measured from the beginning and the end of the study.

### Ingredients and Dosage Calculations

The standard rationed mice's food consists of a mixture of chicken feed (PAR-S produced by JAPFA COMFEED) 66.6% and 33.4% wheat flour which has a calorie content of 2700-2800 kcal /kg given as much as 70-80 grams/day.[14] Soy milk is made with a ratio of 20 grams of soybean powder and 160 ml of distilled water. The concentration of the solution is 0.125g/ml. Soybean powder uses Fressoya with license number Food-Home Industry (P-IRT) 815350701862 which is produced by CV. Fresco Food Industry. Genistein content in soybean powder is 4.4 mg / g. Genistein is a solution with a concentration of 0.5mg / ml. Pure genistein is produced by the Wuhan Economic and Technological Development Zone, Wuhan, Hubei with number CFN98681.

The treatment dosage is based on human genistein consumption in Asian race (standard weight 60 kg) is 20-80 mg/day, with low dose (20 mg/day), medium dose (40 mg/day) and high dose (80 mg/day).[1] The dose is converted into a dose in mice with the formula: mice dose (mg/kg)= human dose /day (mg/kg) x 6.2 (constant).[15] Then, the conversion for low, medium and high doses was obtained of 0.4 mg, 0.8 mg, and 1.6 mg/200 g mice weight /day.

Soy milk supplementation that was given was 0.8ml, 1.6ml, and 2x1.6ml for each treatment group with low (K2), medium (K3) and high (K4) doses. While the genistein supplementation given was 0.8 ml, 1.6 ml and 2x1.6 ml for low (K5), medium (K6) and high (K7) doses.

### Pancreatic Histopathology Examination

Mice were sacrificed on the 61st day. The pancreas was fixed in 10% formalin buffer and made paraffin blocks. Histopathological preparations were done by staining with hematoxylin and eosin. Observation of the degree of pancreatic fat infiltration using the modified Papacio and Dembinsky scoring in **Table 1**.[16, 17]

**Table 1**. Degree of fat infiltration

| Score | Fat Infiltration |
|---|---|
| 0 | No vacuolization of acinar cells |
| 1 | <25% vacuolization of acinar cells |
| 2 | 25% -50% vacuolization of acinar cells |
| 3 | > 50% vacuolization of acinar cells |

### Statistical Analysis

Box plots of the subject's baseline and final body weight were used to determine the existence of extreme values. Subjects with extreme body weight values were not used for further statistical analysis. Weight gain, body length and BMI in each group were analysed using a paired T-test. The effect of treatment on body weight and BMI of mice was tested using One Way ANOVA. Analysis of the degree of pancreatic fat infiltration, Kruskal-Wallis test, and if a significant effect was found, it was continued with the Mann-Whitney test for each treatment on the control. The software used for the analysis was IBM © SPSS © version 26.

## RESULTS

In this study, there are four subjects were excluded due to death, which is one in group 2, two in group 5, and one in group 6. Subjects who had extreme body weight values at the beginning and end of the study were not used in the statistical analysis. The characteristics of the research subjects are described in **Table 2**. In this study, the total number of samples used for statistical analysis was 25 samples. Sample characteristics in **Table 2**. One-Way Annova test with Tukey HSD post-hoc test showed no significant difference in final body weight, body length and BMI between control and treatment groups.

Histopathological observations are presented in **Figure 1** and followed by a frequency graph to see the trend of changes that occur in each treatment. Statistical tests using the Kruskall Wallis method showed no significant difference in the infiltration of pancreatic fat cells in the supplementation of soy milk or sub chronic genistein ($p > 0.05$). However, there was a trend of decreasing fat infiltration in the pancreas in the treatment of subchronic soy milk and genistein compared to controls. One-way ANOVA test results showed the effect of giving soy milk and genistein on body weight ($p = 0.022$). However, the results of the post hoc test with Tukey HSD showed that there was no effect of giving soy milk on body weight and BMI between the control and treatment groups at all dose

**Table 2**. Baseline Characteristics of Subjects

| Karakteristik | K1 | K2 | K3 | K4 | K5 | K6 | K7 |
|---|---|---|---|---|---|---|---|
| Total (n) | 3 | 4 | 4 | 3 | 3 | 4 | 4 |
| Initial Weight | 166.9±1.4 | 201.3±10.9 | 219.0± 5.3 | 202.5±10 | 186.7±7.3 | 189.8±7.1 | 206.4±5.4 |
| Final Weight | 325.4±8.7 | 334.2±9.4 | 344.1±15.9 | 333.8±1.4 | 263.6±18.7 | 329.3±15.4 | 344.6±13.4 |
| Initial Length | 16±0.2 | 17.9±0.2 | 17.7±0.13 | 17.9±0.2 | 17.8±0.2 | 17.42±0.5 | 18.07±0.21 |
| Final length | 20.4±0.08 | 20.3±0.2 | 20.6±0.2 | 20.03±0.1 | 19.7±0.5 | 20.2±0.1 | 20.5±0.1 |
| Initial BMI | 0.65±0.02 | 0.62±0.02 | 0.69±0.02 | 0.63±0.03 | 0.59±0.01 | 0.62±0.02 | 0.63±0.01 |
| Final BMI | 0.78±0.05 | 0.8±0.02 | 0.81±0.04 | 0.83±0.01 | 0.67±0.02 | 0.81±0.03 | 0.81±0.02 |

** Description: Data is presented in the form of Mean + SEM (Standard Error of Mean); BMI, body mass index*

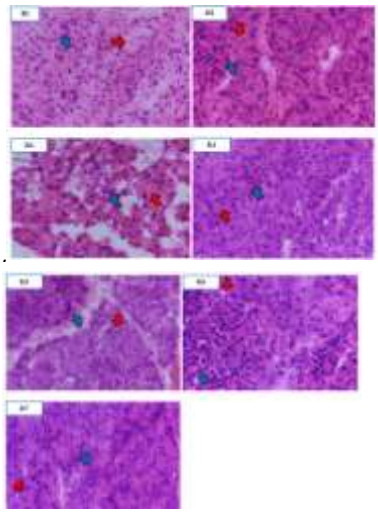

**Figure 1**. Comparison of the histopathological features of the pancreas in various treatment groups. The red arrow indicates the presence of fat micro-droplets (fat deposits) in the pancreatic acini. The blue arrow indicates the presence of inflammatory cell infiltration (fibrosis cells). H.E. 400x

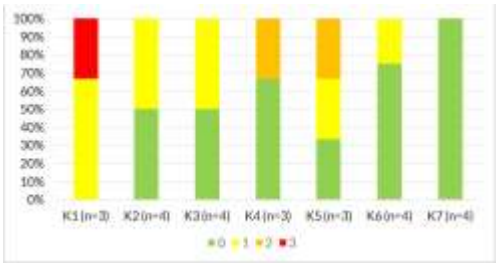


**Figure 2**. Degree of pancreatic fat infiltration in each treatment group (p = 0.204)

Each group showed significant weight gain in the K2 (p=0.025), K4 (p=0.029), K6 (p=0.017), and K7 (p=0.014) groups, this is consistent with the increase in BMI in the K2 group (p=0.025), K4 (p=0.029), K6 (p=0.017) and K7 (p=0.014). Groups K2 (0.8 mL of soy milk) and

K4 (3.2 mL), which are groups with small doses of soy milk and large doses equivalent to 2.1 and 8.3 mg/kg/day for mice or equivalent 20 and 80 mg/day for human doses (bb = 60kg). In the K6 and K7 groups the dose was equivalent to the daily consumption of genistein 4.1 and 8.3 mg / kg / day which is equivalent to the dose of 40 and 80 mg / day for the human dose (body weight = 60kg).

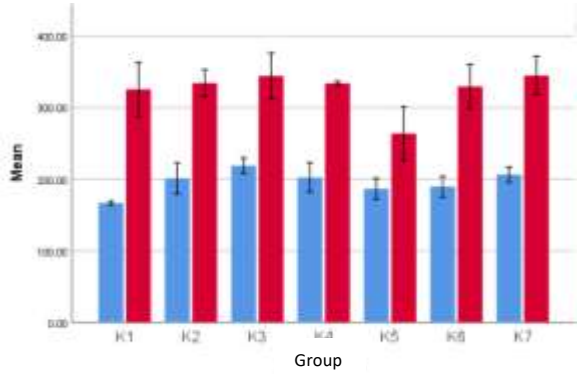


**Figure 3**. Figure of body weight of each treatment group. Blue: initial weight, Red: final weight.

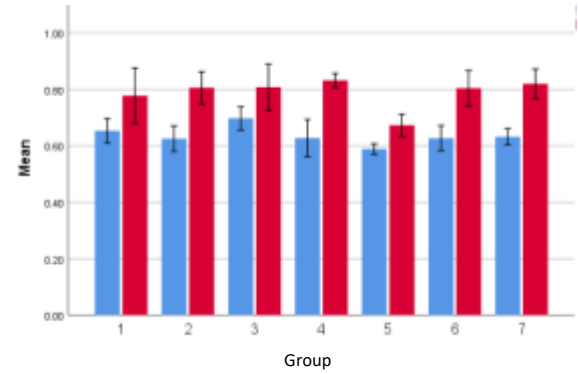


**Figure 4**. Figure of BMI in each treatment group. Blue: initial BMI, Red: final BMI. BMI, body mass index

## DISCUSSION

In this study, 4 subjects were excluded due to death. Each of them is 1 rat in the soy milk group and 3 in the genistein group. The proportion of sample deaths reached 11.3%, exceeding the initial estimate of 10%. In group 5, there were 2 mice that died so that the number of samples was only 3, less than the minimum sample requirement in the calculation of the federer's formula.

Giving soy milk and genistein did not significantly affect differences in body weight and BMI between control and treatment groups at all doses. However, weight gain in the soy milk and genistein groups was not consistent with previous studies. Research by Wang, *et al*. shows that giving large doses of soy milk and genistein supplementation in male mice tends to lose weight. Whereas at low doses it tends to increase body weight. However, there is a gap between invitro and in vivo studies due to the overlapping of transcription factors, metabolism and the stability of active substances.[18] The use of soy milk and genistein also gave different results, because in soy milk there is another isoflavone, daidzein, which affects lipogenesis via the PPAR-α independent pathway in hepatocytes rather than via estrogen receptors in adipocyte tissue. Microarray gene analysis, showed adipogenesis in low-dose genistein was influenced by the phospholipase A2 group 7 gene and phospholipid transfer protein. Meanwhile, the anti-adipogenic activity of genistein and down-regulation of the adipogenic gene require expression of ERβ.[19]

From **Table 2** it can be seen that all groups of mice are obese. Normal BMI in adult male mice ranges from 0.45 to 0.68 g / cm2.[20] This result is not in accordance with the initial study design, because the control used is a negative control, so that obesity is not expected in the control group. This study did not calculate the amount of leftover feed, and the physical activity of the mice. Obesity that occurred in all groups in this study could occur due to excess calorie intake compared to daily calorie requirements compared to physical activity of mice.[21] Daily calorie requirements of Sprague Dawley male mice are 110 kcal ME/$BW_{0.75\ kg}$/day.[22] While the food intake given in this study in all groups was 70-80 g/cage/day which was equivalent to 120-137 kcal/rat/day.

In this study, the control group contained mild to severe pancreatic fat infiltration. This indicates that the control group occurred an ectopic fat infiltration process. Ectopic fat infiltration is preceded by inflammation of the acinar cells, followed by acinar cell death and replaced by adipocyte droplets.[11] This figure is not in accordance with the initial hypothesis of the study, namely that the negative control is expected to have no picture of pancreatic fat infiltration. The presence of pancreatic fat infiltration in the control group, seems to be related to the incidence of obesity in the control group. BMI at the time of the surgery will be performed the control group was 0.78±0:05 g/cm². Normal BMI in mice ranged from 0:45 to 0.68 g/cm2.[20] In male sex, decreased expression of ERα due to the absence of estrogen

stimulation causes the distribution of adipocyte tissue to go to the visceral organs. [8, 23]

Research by Ahmad (2014), concluded that feeding the standard chicken feed to mice significantly increased body mass and liver compared to the group with standard laboratory feed. In mice that were given chicken feed also found obesity. An increase in liver weight indicates the production of excess protein and fat so that the liver cells hypertrophy. This is due to the high levels of cholesterol, amino acids and fat precursors in the feed. The study also compared feeding with pure soybeans, where pure soybeans significantly reduced rat body weight compared to standard lab feed, as well as chicken feed.[24] In this study, there was no effect of weight loss from supplementation of soy milk or genistein.

The absence of a trend in weight loss in this study is due to the different gut microbiota of mice from humans. Intestinal absorption of genistein is the main prerequisite for genistein to work. Bacteria contained in the small intestine of mice can change the structure of β-glucosides. However, because genistein is stable in the intestinal lumen, it is difficult to change the structure of β-glucoside from genistein to genistein during hydrolysis.[25] Isoflavones in the form of glycosides cannot be completely absorbed by intestinal cells and their bioavailability requires initial hydrolysis by the β-glucosidase enzyme to then be carried to the peripheral circulation.[26] In rat intestinal tissue, genistein (isoflavone in the form of glycosides) and / or its metabolites are not absorbed.[27] This study did not assess these differences in microbiota.

The results of this study showed that there was no significant difference in the effect of giving soy milk and genistein compared to control on pancreatic fat infiltration ($p=0.204$). However, the trend showed a decrease in fat infiltration in the soy milk and genistein groups and was directly proportional to the dose of soy milk and genistein. This may be influenced by the function of soy milk isoflavones and genistein which function as selective ligands for ERβ. This is because the affinity of these phytoestrogens to ERβ is 20-30x higher than ERα.[28]

Expression of adipocyte tissue ERβ functions to reprogram preadipocytes and mesenchymal stem cells to turn into *brown fat* (BAT) and increase mitochondrial respiration. This expression also increases energy biogenesis and oxygen consumption from the pathway's *tricarboxylic acid-dependent* and *independent*. The cumulative effect that can occur is a reduction in adipocytes and body weight.[29] However, this pathway was not seen in this study. Because there are no significant differences and trends in weight loss and BMI.

Factors that can influence the trend of decreasing pancreatic fat infiltration are the anti-inflammatory effects of isoflavones in soy milk and genistein. Visceral fat, tends to release adipokines and pro-inflammatory mediators in large quantities, which will trigger insulin resistance, increase triglyceride lipolysis and release free fatty acids into the circulation.[11] Genistein increases the potency of AMPK (AMP-activated protein kinase) / mitofucine 2 activation in preadipocytes and *white / brown adipocytes* thereby protecting them from the effects of hydrogen peroxidase by inhibiting ROS and maintaining mitochondrial function.[30]

Estrogen receptor modulation is only one pathway in the cascade of adipocyte tissue function and metabolism. The phenotype of adipocyte tissue is the resultant of a variety of other metabolic pathways, and is influenced

by gender and dose, as well as length and time of exposure, of isoflavones and genistein.

In vitro studies, concluded that genistein can cause adipogenesis through disruption of estrogen receptors (ERs). Exposure to genistein given inhibits differentiation of human adipocytes cells with the *down* regulation of ERα (doses of 25 and 50 m) and ERβ (6:25 and 25 m). The biggest decrease that occurred was in Erβ. The dosage in this study was very high when compared to 6.6 μM for infant soy formula and 2.4 μM for soybean powder in adults.[31] However, there is also a biphasic effect, that is, at low doses it tends to produce antiadipogenic effects. This effect occurs when genistein is given in the early phase of adipocyte differentiation, and has a long-term effect on the reduction of tissue volume of adipocytes.[18]

In animal studies, the effects arising from the administration of soy milk isoflavones (geneistein, daidzein, glycetein), consistently reduce obesity and improve lipid metabolism profiles in male and female animals. Fat deposits in the visceral organs were also decreased in the high-fat and normal model diets. But the biphasic effect still appears in male experimental animals. Low doses of genistein appear to increase visceral fat, but at high doses (200 mg/kg /day) reduce visceral fat. The difference between in vitro and in vivo studies is due to the *overlapping* roles of transcription factors and metabolic factors, as well as the stability of the active substances used.[18]

Human studies show changes in fat distribution in menopausal women. This shows that the action of soy milk isoflavones is closely related to ERs. Administration of soy milk isoflavones and their derivatives also upregulated anti-inflammatory genes at all doses. However, the biphasic effect is still visible, namely at low doses there is a downregulation of genes that express fat energy consumption. Further studies have shown the role of the gut microbiota in converting isophavones into active metabolites. In patients with an obesity profile, 2.8x were less likely to produce active metabolites of isoflavones in the intestine.[18]

Another effect seen in human studies is a change in fat distribution from visceral to subcutaneous. The pathway of change is through increased lipoprotein lipase activity. Another important pathway is the reduction of inflammation through *downregulation of* IL-6 through its transcription factor, NF-kB, (this substance plays a major role in *downregulation* of estrogen receptors).[18]

In this study, we suspect the trend of decreasing degree of pancreatic fat infiltration is due to the anti-inflammatory effects of soy milk and genistein. The effect will prevent the *down* regulation of the estrogen receptor, so that isoflavones and genistein soy milk can induces estrogen receptors on adipocytes network, presence of hormone stimulation modulation of estrogen receptors on adipocytes male network will improve the function and tissue distribution of adipocytes.

## Limitations of the Study

These are limitations of the study1) The number of samples is too small because the number of deaths exceeds the estimated number and the existence of data with extreme values that are not included in the statistical analysis. 2) The content of other isoflavones in soy milk was not checked. The examination is needed because the isoflavone compound other than genistein in soy milk is a phytoestrogen which can become EDC on the function and distribution of adipocytes. 3) The negative control in this study is obesity and

there is an infiltration of fat in the pancreas due to factors beyond the knowledge of researchers that were not considered at the time of the initial study design. 4) This study did not use estrogen treatment as a positive control as a comparison of the endocrine disruption effect of the studied phytoestrogens. 5) Confounding factors such as gut microbiota were not assessed.

## CONCLUSION

The supplementation of subchronic soy milk and subchronic genistein did not significantly reduce the pancreatic fat infiltration of Sprague Dawley male mice. There was no difference in the degree of pancreatic fat infiltration of male Sprague Dawley mice in the supplementation group with soy milk and subchronic pure genistein.

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