Journal of Animal Science and Technology 52(5) 383~388, 2010
DOI:10.5187/JAST.2010.52.5.383

Extracts of Korean Medicinal Plant Extracts Alter Lipogenesis of Pig Adipose Tissue and Differentiation of Pig Preadipocytes *In vitro*

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한국 약용식물 추출물이 *In vitro* 돼지 지방조직의 지방합성과 지방전구세포의 분화에 영향을 미친다

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요 약

In vivo 실험에 의해서 지방축적을 억제할 수 있는 물질을 찾아내는 것은 시간과 비용이 많이 든다. 본 연구는 돼지 지방조직을 이용한 *in vitro* system을 이용해서 한국의 약용식물 중에서 지방축적 억제 작용을 가지는 것을 조사하고자 수행하였다. 총 183종의 약용식물을 이용하여 이들이 돼지 지방조직의 지방합성과 지방전구세포의 분화에 미치는 작용을 조사하였다. 에탄올 추출물은 72종, 물 추출물은 111종, 이 중에서 65종은 물과 에탄올 모두 이용해서 추출하였다. 돼지 지방조직의 지방합성은 13종류의 약용식물이 영향을 미쳤는데, 그 중 11종은 대조구에 비하여 지방합성을 40% 이상 억제하였고, 4종은 지방합성을 70% 이상 억제하였다. 가장 강력한 지방합성 억제작용을 나타낸 것은 붓꽃과와 고삼(에탄올 추출) 그리고 좁쌀풀(물과 에탄올 추출)이었다. 그러나 소목과 황백은 지방합성을 촉진하였다. 돼지 지방전구세포의 분화에 미치는 결과는 총 28종의 약용식물이 돼지 지방전구세포의 분화에 영향을 미쳤는데, 이 중 16종의 약용식물은 증가시켰고, 12종은 억제시켰다. 목단피와 강활(에탄올 추출) 그리고 당귀, 목향 및 신이(물 추출)는 지방세포의 분화를 두 배 정도 증가시켰다. 10종의 약용식물 즉 감초, 형개 및 구월나무(에탄올 추출) 그리고 비름과, 천문동, 백출, 유자나무, 향부자, 구엽초 및 목단피(물 추출)은 지방세포의 분화를 35% 이상 억제시켰다. 구월나무(에탄올 추출)만이 지방합성뿐만 아니라 세포분화도 억제하였다. 본 연구 결과는 약용식물로부터 가축과 사람의 지방축적 억제효능 후보물질을 발굴하기 위한 기초자료로 활용할 수 있을 것으로 여겨진다.
(**Key words :** Medicinal herbs, Lipogenesis, Adipocyte differentiation, Pig)

INTRODUCTION

Accumulation of excessive fat in livestock reduces the economic efficiency of meat production. Obesity in human is a serious risk to health (Ahima, 2006; Huang et al., 2009). Therefore there is great interest in agriculture and medicine in the causes, diagnosis, treatment and/or prevention of obesity (Adeneye et al., 2010; Lee et al., 2010; Rayalam et al., 2008). At present there is no simple genetic predictor of obesity in human or livestock because of the complex polygenic origin of this condition (Herd et al., 2003) and there exists no practical therapy to reduce obesity (Cheetham et al., 2004). The major focus is therefore on prevention (Skidmore and Yarnell, 2004). Pharmacological strategies can be successful in preventing obesity, but are not suitable for meat production due to cost effectiveness (Dunshea et al., 2003). Another approach that has practical promise in preventing obesity is dietary including both macronutrient and micronutrient constituents of food. Use of medicinal herbs is an attractive alternative because of their relatively low cost. Reduction in obesity requires either reduction in the rate of lipogenesis by adipocytes or a reduction in the number of mature adipocytes present in fat depots. Identification of dietary micronutrients or other natural products that have these abilities and can prevent the development of obesity *in vivo* is time consuming and expensive. We have used two *in vitro* systems derived from pig adipose tissue to screen simple aqueous or alcohol extracts from a large number of traditional Korean medicinal

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herbs for their anti-adipogenic potential. One system used slices of adipose tissue from mature pigs to measure rates of synthesis of lipids. The other system used primary cultures of preadipocytes isolated from newborn pigs to measure rates of differentiation into mature adipocytes. This study has identified Korean medicinal herbs that may reduce fat deposition.

MATERIALS and METHODS

1. Extraction of traditional Korean herbal medicines

Dried "herbs" were purchased at the Kyung-Dong market, Seoul, Republic of Korea. They were extracted by addition of one litre of either ethanol (Table 1) or water (Table 2) to 100 gram dry weight and sonication for 4 hours at 25ºC. The insoluble residue was recovered by filtration and sonicated as above in an additional litre of the same extraction solvent. The insoluble material was removed by filtration. The first and second filtrates were combined and evaporated to dryness under vacuum at 50ºC. The dried extract was then powdered and stored at − 20ºC. Each extract was dissolved in extraction solvent, i.e.ethanol or water, to prepare working stocks of 10 mg/ml prior to testing of biological activity.Four replicates of each extract were tested in each of the following *in vitro* bioassays. Extraction solvent was used as the control (blank) for each assay.

2. Lipogenesis in slices of adipose tissue from adult pig

Specimens of ~5 g of dorsal adipose tissue were obtained from female crossbred Yorkshire × Landrace × Duroc pigs of ~100 kg live weight and transported to the laboratory at 37 ºC in a sterile solution of Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis MO, D-5523). Under aseptic conditions, adipose tissue was sliced to pieces of ~15 mg and incubated for 48 h at 37ºC in DMEM containing 0.3 μg/ml insulin (Sigma, I-664), 0.5 μg/ml transferrin (Sigma, T-1283), 2.5 μg/ml hydrocortisol (Sigma, H-0396) and 50 μg/ml plant extract (Table 1). The ~15 mg adipose tissue slices were recovered and each was added to a glass vial containing 3 ml of sterile Krebs Ringer bicarbonate buffer containing 0.3 μg/ml insulin, 5 mmol/l D-glucopyranose 6-phosphate (Sigma, G7879) and 0.5 μCi D-[U-^{14}C]glucose (GE Healthcare Bio-Sciences, Little Chalfont, United Kingdom, CFB96) and incubated at 37ºC for 2 h under a mixture of 5% CO_2 and 95% O_2. The vials were placed on ice for 30 min. Adipose tissue slices were recovered, weighed and placed in a fresh glass vial containing 5 ml Dole's solution (Dole, 1956), a mixture of 40 parts isopropanol, 10 parts n-heptane and 1 part 1N H_2SO_4 (by volume), and sonicated

Table 1. Species from which ethanol soluble extracts were tested
(Entries in boldface indicate water soluble extracts were also prepared)

Acorus gramineus	Ledebouriella seseloides
Amaranthaceae	Lycium chinense
Anemarrhenae rhizoma	Lysimachia vulgaris L
Angelica acutiloba	Magnoliae flos
Angelicae gigantis radix	**Maranthaceae**
Araliaceae	Moutan radicis cortex
Araliae cordatae radix	Nelumbo mucifera
Astragali radix	Nepetae spica
Atractylodis rhizoma	Ostericum koreanum
Atractylodes japonica	Paeonia japonica
Bombycis corpus	Paeonia lactiflora
Bupleuri radix	Palmales
Caprifoliaceae	Panax pseudo-ginseng
Cinnamomi cortex	Persicae semen
Cimicifuga foetida L.	Phellodendri cortex
Cimicifugae rhizoma	Pinellia ternata
Citrus junos TANAKA	Polygala myrtifolia
Citrus unshiu	Ponciri fructus
Coicis semen	Prunella vulgaris L.
Corydalis turtschaninovii	Prunus persica
Cratae pinnatifida BUNGE	Prunus salicina KINDL
Cyatheaceae	Pueraria thunbergiana
Darthamus tinctorius L.	Pulsatilla koreana
Dryopteris crassirhizoma NAKAI	Ranunculaceae
	Rehmannia glutinosa
Ephedra sinica	Rutaceae
Eucommiaceae	Salvia miltiorrhiza
Gardenia jasminoides for.	Schizonepeta tenuifolia var.
Gastrodia elata BLUME	Scrophularia buergeriana MIQ
Gleditsia sinesis LAM	Sophora flavescens AIT
Glycyrrhizae radix	Stephania tetrandra S.
Glycyrrhize uralensis	Tagetes patula L.
Gnetopsida	Taraxacum platycarpum
Gupressaceae	Trichosanthes kirilowii MAX
Hoelen	Viscum coloratum
Houttuynia cordata	Zingiberis rhizoma
Iridaceae	

Table 2. Species from which water soluble extracts were tested
(Entries in boldface indicate ethanol soluble extracts were also prepared)

Acorus gramineus	**Crataegi fructus**	**Lespedexa pilosa**	Polygala myrtifolia
Alismatis rhizoma	Cratae pinnatifida BUNGE	**Linderae radix**	**Polygalaceae**
Amaranthaceae	**Cucumis sativus L.**	**Liriopis tuber**	**Polyporus**
Anemarrhenae rhizome	**Cupressaceae**	**Lycii fructus**	Ponciri fructus
Angelicae gigantis radix	Cyatheaceae	Lycium chinense	**Poria cocos**
Atractylodis rhizoma	**Cyperi rhizome**	Lysimachia vulgaris L.	Prunella vulgaris L.
Anthrisci radix	**Cyperus rotundus**	**Magnoliae cortex**	Prunus persica
Araliaceae	Darthamus tinctorius L.	**Magnoliae flos**	Prunus salicina KINDL
Araliae cordatae radix	Dryopteris crassirhizoma	**Mentha arvensis L.**	**Psoraleae semen**
Artemisiae asiaticae herba	NAKAI	**Menthae herba**	**Puerariae radix**
Asparagus cochinchinesis	Ephedra sinica	Moutan radicis cortex	Pueraria thunbergiana
Astragali radix	**Epimedium grandiflorum**	**Natrii sulfas**	**Pulsatilla koreana**
Atractylodes japonica	**Equiseti herba**	Nelumbo mucifera	Ranunculaceae
Atractylodis rhizoma alba	**Eucommia ulmoides**	Nepetae spica	Rehmannia glutinosa
Bombycis corpus	**Fritillaria verticillata**	**Notoginseng radix**	Rutaceae
Bupleuri radix	Gardenia jasminoides for.	Ostericum koreanum	Salvia miltiorrhiza
Caesalpiniae lignum	Gastrodia elata BLUME	Paeonia japonica	Schizonepeta tenuifolia var.
Caprifoliaeae	Gleditsia sinesis LAM	Paeonia lactiflora	**Scrophularia buergeriana MIQ**
Cimicifugae rhizoma	Glycyrrhizae radix	Palmales	**Scutellaria baicalensis**
Cinnamomi cortex	Glycyrrhize uralensis	**Panix ginseng**	Sophora flavescens AIT
Cinnamomi ramulus	Gnetopsida	Panax pseudo-ginseng	Stephania tetrandra S.
Citrus junos TANAKA	**Gypsum**	**Perillae herba**	Tagetes patula L.
Citrus unshiu	Hoelen	Persicae semen	Taraxacum platycarpum
Cnidium officinale	**Hordei fructus germinatus**	Phellodendri cortex	**Terminaliae fructus**
Codonopsis pilosula	**Houttuynia cordata**	**Phellodendron amurense**	Trichosanthes kirilowii MAX
Coicis semen	**Inula helenium L.**	**Phyllostachys nigra**	Viscum coloratum
Corydalis tuber	**Lardixabalaceae**	Pinellia ternata	Zingiberis rhizoma
Corydalis turtschaninovii	Ledebouriella seseloides	**Plantaginales**	**Zingiber officinale**

for 30 min. Hexane (3 ml) was then added and mixed thoroughly. The hexane (upper phase) containing lipid was recovered and placed in a scintillation vial. Residual lipid in the Dole's solution was recovered by addition of 1.5 ml of hexane and extraction repeated as above. Scintillation fluid (PerkinElmer Life and Analytical Sciences Inc., Wellesley MA, Ultima Gold 6013329) was added to the combined hexane extracts and the ^{14}C-radioactivity in the hexane extracted lipids was measured in a liquid scintillation spectrometer. Lipogenesis was measured in replicates of 4. Data is presented as mean ± standard error of the mean.

3. Isolation of porcine preadipocytes

Porcine preadipocytes were isolated from the stromal-vascular fraction of dorsal subcutaneous adipose tissue (Suryawan et al., 1997). Female piglets were obtained from a commercial producer on the day of birth or the following day and killed by CO_2 asphyxiation. Their skin was immediately scrubbed and washed with an aqueous solution of 20% iodine and 70% ethanol. Dorsal subcutaneous adipose tissue was aseptically isolated, weighed and placed in 3 ml per g of fat tissue of sterile Krebs Ringer bicarbonate buffer containing 20 mmol/l HEPES (4-[2-hydroxyethyl]piperazine-1-ethanesulfonic acid, Sigma, H-4034), 200 mg/l gentamycin sulphate (Sigma, G-1264) and 2,000 U per g fat tissue of type-1 collagenase (Worthington Biochemical Corporation, Lakewood, NJ, LS004196) and finely minced with sharp sterile scissors. Minced adipose tissue was digested with collagenase for 40 min at 37ºC. Undigested tissue and suspended cells were separated by filtration through a 250 μm nylon mesh. Isolated cells were recovered in the filtrate and pelleted by centrifugation at

3,000 rpm for 10 min. The supernatant was discarded. The cells were washed by resuspension in Krebs Ringer bicarbonate buffer and centrifugation at 2,000 rpm for 10 min. The supernatant was discarded and the cell pellet was resuspended to 20 ml final volume withplating media consisting of 10% fetal bovine serum (Gibco Life Technologies, Grand Island NY, 16000-044) in a 50/50 mixture of DMEM and Ham's F-12 (Sigma, N-6760) containing 12 mmol/l HEPES and 50 mg/l gentamycin sulphate. The cells were again filtered through a 250 μm nylon mesh. Cells were counted using a hematocytometer and seeded in a 6-well plate (Corning Costar 3506) at a density of 6×10^6 cells in 2 ml per well and incubated at 37ºC under a mixture of 5% CO_2 and 95% O_2.

4. Differentiation of adipocytes

Twenty-four hours after seeding, primary cultures of porcine preadipocytes were washed three times with plating media without fetal bovine serum. This was designated as day 0 of culture. Cells were subsequently cultured in the same media containing 0.3 μg/ml insulin, 0.5 μg/ml transferrin, 2.5 μg/ml hydrocortisol and 5% fetal bovine serum. Media was replaced every second day for 14 days. Plant extracts (Table 1) were included in the media at various concentrations from day 0 to day 4. The extent of cell differentiation was assessed on day 14 by spectrophotometric assay of glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.1.8) by measuring the disappearance of NADH during GPDH catalyzed reduction of dihydroxyacetone phosphate (Wise and Green, 1979). Differentiation was measured in replicates of 4.

RESULTS AND DISCUSSION

A total of 183 extracts of Korean medicinal herbs (KMH) were tested. Ethanol extracts were prepared from 72 different medicinal herbs (Table 1). Aqueous extracts were prepared from 111 medicinal herbs (Table 2). Both an ethanolic and an aqueous extract were prepared from 65 of these medicinal herbs. Thirteen extracts substantially altered rates of lipogenesis *in vitro* (Table 3). Six were aqueous and seven were ethanolic extracts. Most of the active extracts were found to reduce the rates of lipogenesis in adipose tissue slices. Eleven of the active extracts reduced lipogenesis to rates that were more than 40% lower than control and fourof these reduced rates of lipolysis by more than 70%. The most potent anti-lipogenic extracts were the ethanolic extracts obtained from *Iridaceae* and from *Sophora flavescens AIT* as well as both the aqueous and ethanolic extracts from *Lysimachia vulgaris L.* Two extracts, those prepared in water from *Caesalpiniae lignum* and from *Phellodendri cortex*, were found to promote rates of lipogenesis *in vitro* (Table 3).

Twenty-eight extracts altered the rates of differentiation of cultured porcine preadipocytes (Table 4). Eleven were ethanolic and 17 were aqueous extracts of traditional Asian medicines. Sixteen of these extracts increased and twelve reduced the rates of differentiation of preadipocytes. Extracts prepared in ethanol from *Moutan radicis cortex* and from *Ostericum koreanum* and those prepared in water from *Angelicae gigantis radix*, from *Inula henenium L* and from *Magnolia flos* doubled the rate of differentiation of cultured porcine preadipocytes. Ten extracts were found to reduce the *in vitro* rate of differentiation of porcine preadipocytes by more than 35%. These were the ethanolic extracts from *Glycyrrizae radix, Nepetae spica* and from *Polygala myrtifolia* and the aqueous extracts from *Amaranthaceae, Asparagus cochinchinesis, Atractylodis rhizoma alba, Citrus junos TANAKA, Cyperus rotundus, Epimedium grandiflorum* and from *Moutan radicis cortex*. Only the ethanolic extract from *Polygala myrtifolia* was able to both reduce lipogenesis in adipose tissue slices and retard differentiation of cultured preadipocytes.

There have been recent reports that some plant extracts decrease differentiation of the 3T3-L1 cell line derived from

Table 3. Effects of extracts of medicinal herbs on *in vitro* lipogenesis of pig adipose tissue

	Percentage of control (Mean ± SD)	Extract
Artemisiae asiaticae herba	40.6 ± 7.8	water
Bombycis corpus	36.2 ± 9.0	water
Caesalpiniae lignum	146.9 ± 12.6	water
Corydalis turtschaninovii	41.9 ± 1.1	ethanol
Iridaceae	21.6 ± 1.9	ethanol
Lysimachia vulgaris L	27.5 ± 1.3	ethanol
Lysimachia vulgaris L	24.7 ± 18.8	water
Phellodendri cortex	136.7 ± 13.0	water
Phellodendron amurense	47.0 ± 8.0	water
Polygala myrtifolia	41.5 ± 4.7	ethanol
Prunus salicinia	41.3 ± 3.7	ethanol
Sophora flavescens AIT	25.8 ± 7.4	ethanol
Trichosanthes kirilowii MAX	47.9 ± 11.9	ethanol

Table 4. Effects of extracts of medicinal herbs on *in vitro* differentiation of pig preadipocytes

	Percentage of control (Mean ± SE)	Extract
Acorus gramineus	129.0 ± 3.3	ethanol
Amaranthaceae	45.9 ± 5.4	water
Anemarrhenae rhizome	139.4 ± 18.0	water
Angelicae gigantis radix	220.8 ± 9.6	water
Araliaceae	141.8 ± 5.9	ethanol
Asparagus cochinchinesis	56.3 ± 4.0	water
Atractylodis rhozoma alba	54.1 ± 2.9	water
Cinnamomi cortex	161.1 ± 7.5	ethanol
Citrus junos TANAKA	145.6 ± 5.9	ethanol
Citrus junos TANAKA	51.0 ± 0.2	water
Citrus unshiu	173.1 ± 12.0	water
Crataegi fructus	64.1 ± 8.2	water
Cyperus rotundus	59.4 ± 2.9	water
Epimedium grandiflorum	59.8 ± 5.4	water
Glycyrrizae radix	59.5 ± 4.7	ethanol
Houttuynia cordata	129.2 ± 2.9	water
Inula henenium L	233.3 ± 9.8	water
Magnoliae flos	233.5 ± 19.4	water
Nepetae spica	59.4 ± 2.0	ethanol
Notoginseng radix	136.7 ± 7.3	water
Ostericum koreanum	202.5 ± 11.1	ethanol
Perrillae herba	67.1 ± 5.7	water
Phellodendron amurense	139.9 ± 7.6	water
Polygala myrtifolia	55.9 ± 5.0	ethanol
Moutan radicis cortex	282.1 ± 5.3	ethanol
Moutan radicis cortex	57.1 ± 5.4	water
Sophora flavescens AIT	128.5 ± 8.2	ethanol
Stephania tetrandra S	145.2 ± 7.7	ethanol

mouse or primary preadipocytes of rat (Ogawa et al, 2010; Popovich et al, 2010). However, the results of our studies which used primary cultures of pig preadipocytes provide more valuable information regarding the suitability of such extracts for reduction of fat deposition in livestocks and humans. The reason for this is the difference in the physiology of adipogenesis between the rodents and other mammals. One example to support this is that the blood level of the insulin like growth factor-II which has been known to be involved in fat deposition of growing pigs (Owens et al, 1999) is high in the pig and is very low in the rat postnatally (Moses et al, 1980; Lee et al, 1991). Our current study is the first to investigate the effects of Korean medicinal herbs on lipogenesis and differentiation of pre-adipocytes.

The results of the current study showed that many of Korean medicinal herbs decreased lipogenesis of adipose tissue and differentiation of pig preadipocytes *in vitro*. However, it should be noted that the actions shown by *in vitro* method may not be replicated *in vivo*. All Korean medicinal herbs that showed anti-adipogenic action in our *in vitro* study need to be tested.

ABSTRACT

Identification of natural compounds that can prevent the development of obesity *in vivo* is time consuming and expensive. We have used *in vitro* systems derived from pig adipose tissue to screen simple aqueous or ethanolic extracts of Korean medicinal herbs (KMH) for their anti-adipogenic potential. A total of 183 extracts were tested for their actions in lipogenesis of pig adipose tissue and differentiation of pig preadipocytes. Ethanol extracts were prepared from 72 and aqueous extracts were prepared from 111 medicinal herbs. Both an ethanolic and an aqueous extract were prepared from 65 of these. Thirteen extracts substantially altered rates of lipogenesis *in vitro*.

The effects of KMH on lipogenesis of pig adipose tissue are as follows. Elevens reduced lipogenesis to rates that were more than 40% lower than control and four of these reduced rates of lipogenesis by more than 70%. The most potent anti-lipogenic extracts were those obtained in ethanol from *Iridaceae* and from *Sophora flavescens AIT* as well as both the aqueous and ethanolic extracts from *Lysimachia vulgaris L.* Two extracts, those prepared in water from *Caesalpiniae lignum* and from *Phellodendri cortex*, were found to promote rates of lipogenesis *in vitro*. The effects of KMH on differentiation of pig preadoipocytes are as follows. Twenty-eight extracts altered the rates of differentiation of cultured porcine preadipocytes. Sixteen increased and twelve reduced the rates of differentiation of preadipocytes. Extracts prepared in ethanol from *Moutan radicis cortex* and from *Ostericum koreanum* and those prepared in water from *Angelicae gigantis radix*, from *Inula henenium L* and from *Magnolia flos* doubled the rate of differentiation of cultured porcine preadipocytes. Ten extracts reduced the *in vitro* rate of differentiation of porcine preadipocytes by more than 35%. These were the ethanolic extracts from *Glycyrrizae radix*,

Nepetae spica and from *Polygala myrtifolia* and the aqueous extracts from *Amaranthaceae, Asparagus cochinchinesis, Atractylodis rhizoma alba, Citrus junos TANAKA, Cyperus rotundus, Epimedium grandiflorum* and from *Moutan radicis cortex*. Only the ethanolic extract from *Polygala myrtifolia* was able to both reduce lipogenesis in adipose tissue slices and retard differentiation of cultured preadipocytes. The results of our study will provide meaningful information to identify medicinal herbs which would reduce fat deposition in livestocks and humans

(**Key words** : Medicinal herbs, Lipogenesis, Adipocyte differentiation, Pig)

ACKNOWLEDGEMENTS

This research was supported by Technology Development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

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(접수일자 : 2010. 7. 23 / 수정일자 : 2010. 10. 7 /
채택일자 : 2010. 10. 11)