

Effect of White Kwao Extract (*Pueraria Mirifica*) on *in vitro* Development and Implantation Rate of Mouse Embryo

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I. INTRODUCTION

Abstract—The White Kwao (*Pueraria mirifica*), a potent phytoestrogenic medicinal plant, has long been used in Thailand as a traditional folk medicine. However, no scientific information of the direct effect of White Kwao on the development of mammalian embryo was available. Therefore, the purpose of this study was to investigate the effect of White Kwao extract on the *in vitro* development and implantation rate of mouse embryos.

This study was designed into two experiments. In the first experiment, the two-cell stage mouse embryos were collected from the oviduct of superovulated mature female mice, and randomly cultured in three different media, the M16, M16 supplemented with 0.52 µg estrinylestradiol-17β, and M16 supplemented with 10 mg/ml White Kwao extract. The culture was incubated in CO₂ incubator at 37 °C. After the embryos were cultivated, the developments of embryos were observed every 24 hours for 5 days. The development rate of embryos from the two-cell stage to blastocyst stage in the media with White Kwao was significantly higher (p<0.05) than those of the control group (68.50% versus 43.50%) but did not differ from the positive control group (68.50% versus 57.66%).

In the second experiment, hatched blastocysts, which obtained from three different media, were differently labeled the nuclei with two polynucleotide-specific fluorochromes, the propidium iodide (PI) and the bisbenzimidazole. The results showed that the number of trophectoderm cells in the blastocysts that cultivated in the media with White Kwao did not significantly differ from the control (80.00 versus 70 cells) and the positive control group (80.00 versus 112.50 cells). The average number of inner cell mass in the White Kwao treated group did not significantly differ from the control group (20.50 versus 16.00 cells) and the positive control group (20.50 versus 20.50 cells). The total cell number including the trophectoderm and the inner cell mass of the individual hatched blastocyst was evaluated. The cell number in the blastocysts obtained from the media with the White Kwao did not significantly differ from the control (94.25 ± 9.50 versus 92.33 ± 4.05) and the positive control group (94.25 ± 9.50 versus 110.33 ± 9.16).

The results demonstrated that the White Kwao treatment group did have a stimulating effect on the *in vitro* development of mouse embryos. The exact mechanism that White Kwao stimulated mouse embryo development is not known. The suspect mechanism may in a manner similar to the mechanism that of estrogen stimulated the development of the mouse embryos. Further studies are needed to transfer the blastocyst into the endometrium of pseudopregnancy mice to evaluate the effect of White Kwao on implantation.

Keywords—White Kwao (*Pueraria Mirifica*), blastocyst.

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KWAO Khrua (*Pueraria mirifica* Airy Shaw et Suvatabandhu), family *Laguminosae*, subfamily *Papilionoidcae*, is one for the medicinal plants that predominates in the North part of Myanmar, the north part of Thailand such as Chiang Mai, Chiang Rai, Mae Hong Son, Tak and Uthaitani provinces, and the western part such as Rachaburi and Kanchanaburi provinces. It has been used in folk medicine for rejuvenescence by Thai traditional practitioner's medicine for a long time. It was believed that tuberous root of White Kwao is the medicine for long life [1].

The first information of the advantages of using Kwao Khrua was published in a single leaflet, in the 1920s, in the northern part of Thailand, without date or authors. It was discovered at an old Buddhist temple in Paga, in the ancient capital of Myanmar during the temple was reconstructed. There are many types of Kwao Khrua, but they were used only four types in medicine. There are White Kwao, Red Kwao, Black Kwao and Moh Kwao, from these four types, Black Kwao is the strongest and the White Kwao is the weakest potency in rejuvenation. The Kwao Khrua pills are believed to have anti-aging properties such as improve the function of the brain, expand the breast, keep black hair, enhance hair growth on face, nourish the skin, improve memory, prevent the onset of cataracts, improve the sexual efficiency and induce menstruation in women of 60-80 years of age [2]. At the present, the most commonly uses of Kwao Khrua in cosmetics and food supplements are White Kwao and Red Kwao. White Kwao is used for beauty and health in many forms such as tablet, capsule, cream, lotion gel and also used as a hormonal replacement in menopausal woman. The general dose of White Kwao powder of both tablet and capsule are approximately 100-600 mg depending on the manufacture. White Kwao is widely used for relieving menopausal disorders, enhancing breast size, improving memory and prevention of Alzheimer's disease, reducing risk of cataracts, decreasing incidence of colon cancer, eliminating arthritic pain, nourishing skin, and keeping hair stronger [3].

The objectives of the research paper were to evaluate the effect of White Kwao extract on *in vitro* development of pre-implantation mouse embryo.

II. LITERATURE REVIEWS

The tuberous roots of White Kwao accumulates many substances, such as chromenes and coumarins. Chromenes are

importantly active component of White Kwao. These components consist of miroesterol and deoxymiroesterol, they have highest estrogenic activity [4]. Some coumarins in White Kwao such as coumesterol that it has been known as a potent estrogenic activity of coumarins [5].

The important pharmacologic effect of White Kwao is an estrogenic effects. The effect of White Kwao on the development of mammary glands in bilaterally ovariectomized rats and mice showed that White Kwao could stimulate the development of mammary tissue and enlarge the breast size by lengthening and branching the mammary ducts that connect to the nipple [6]. It has been reported that mice feeding with White Kwao could cause vaginal cells maturation or cornification similar to that found in the preovulatory phase. It was possible that White Kwao inhibited the releasing gonadotropins (FSH/LH) by negative feedback on the anterior pituitary gland. In addition, White Kwao was also found to increase uterine weight [7].

Estrogen was recognized as essential for embryonic development and maintenance of pregnancy. The function of estrogen was mediated through its specific estrogen receptor. Using reverse transcription polymerase chain reaction, estrogen receptor (ER-RNA) was detected at the one-cell, two-cell, and four-cell stage. The level became undetectable at the five- to eight cell stages and the morula stage and then reappeared again at the blastocyst stage. The presence of ER mRNA at the blastocyst stage will show estrogen may start to act directly on embryos afterwards and result provide a basis for determining the direct role of estrogen in implantation embryos [8].

In mammals, the fertilization takes place in the ampulla of the oviduct. During the following days, the embryo travels down the oviduct to the uterus, and prepares for implantation. In most of mammalian species, the studies of pre-implantation stage are characterized by a relatively synchronous doubling of cell numbers until the 8-cells stage, followed by a synchronous cell division after compaction. At the 8- to 16-cell stage the embryo enters the uterine environment, develops into a blastocyst, in which the first events of cellular differentiation are observed. At the blastocyst stage the embryo hatches from the surrounding zona pellucid and subsequently implants in the uterine wall [9].

Mouse embryos take about three and a half days to develop from the 1-cell stage to blastocyst stage containing 32 or more cells. The first, 1- to 2-cell and 2- to 4-cell cycles of the mouse embryo take between 16 to 20 hours and 18 to 22 hours respectively, depending on the strain of mice [10].

Compaction is the first event of morphologic and cellular differentiation. The most significant event occurring a compaction is the emergence of distinct cell populations: the blastomere and the trophoblast. The outer cell population of the blastomere develops to form the trophoblast, while the inner cell population is becoming the inner cell mass. The trophoblast cells acquire the characteristics of epithelial cells in being flattened and joined together by tight junction complexes. When the mouse embryo has about 32 cells, trophoblast cells begin to pump fluid intracellular spaces

and later into extracellular spaces, forming the blastocoelic cavity [11]. The blastocyst contains two distinct cell types: the inner cell mass, which go on to form the embryo proper, and the trophoblast cells which are involved in the initial contact with the uterine wall and eventually contribute to the placenta and the extra-embryonic membranes.

The mature blastocyst "hatches" out of the zona pellucida before implanting into the endometrium. Deprivation of estrogen causes a delay in hatching. Although the mechanism of the zona hatching remains to be clarified, these results lead to a hypothetical mechanism by which estrogen stimulates the zona hatching. It may be speculated that estrogen acts on the blastocyst to increase the production of epidermal growth factor receptor on trophoblast cell surface as well as the production of epidermal growth factor in the blastocyst itself and the uterine epithelium. In the trophoblast, the produced epidermal growth factor binds the epidermal growth factor receptor of its own or neighboring trophoblast, by paracrine or autocrine mechanism [12].

III. METHODOLOGY

This experiment was conducted to determine if White Kwao could improve the developmental potency of mouse embryos. The two-cell stage embryos were collected from oviducts of superovulated female mice. These embryos were washed in three changes of PBI medium and finally washed in M16 medium supplemented with 0.4% bovine serum albumin (BSA). Only morphologically normal embryos were randomly divided into three groups, 1. M16 medium as the control group 2. M16 medium containing 0.52 µg/ml ethinylestradiol-17β as the positive control group and 3. M16 medium containing 10 mg/ml lyophilized White Kwao. All of the cultures were placed into 5% CO₂ in air, at 37 °C for 5 days. The developments of the embryos were observed and recorded under inverted microscope every 24 hours for 5 days.

The number of trophoblast (TE) and inner cell mass (ICM) cells of individual hatched blastocysts were counted by differentially labeling the nuclei with two polynucleotide-specific fluorochromes, propidium iodide (PI) was specified TE and bisbenzimidazole was specified ICM. The hatched blastocysts were incubated in H6BSA for a minimum of 20 minutes, and then in TNBS (2,4,6-Trinitrobenzenesulfonic acid) on ice for 10 minutes, and washed in H6 PVP. The embryos were then incubated in rabbit anti-mouse lymphocyte anti-serum for 10 minutes at room temperature. After they were washed in H6 PVP, the embryos were incubated in 23µl of guinea pig supplement (dilute 1:10 H6 BSA) supplemented with 2 µl of propidium iodide (1mg/ml) for 10 minutes at 37 °C, 5% CO₂ in air and washed in H6 PVP. Embryos were fixed in 990 µl of ice-cold absolute ethanol and stained with 10 µl of bisbenzimidazole (2.5 µg/ml). The embryos were left in bisbenzimidazole at 4 °C for three days.

The embryos were mounted by being placed into absolute ethanol for 5-8 minutes. On a glass slide a tiny drop of ultra pure glycerol was placed and an embryo was transferred into the middle of a drop. A cover slip was placed over drop, and

firmly pressed. The embryos were viewed under a fluorescent microscope, and the numbers of nuclei were counted.

IV. FINDINGS

The electro-chemiluminescent immunoassay had been established to measure the estradiol level in M16 media containing lyophilized White Kwao extract at 10 mg/ml, and the others containing ethinylestradiol-17 β 0.52 μ g/ml before the using of media in embryo culture. This analysis was repeated three times respectively. The result showed that the average level of estradiol value of M16 medium contained lyophilized White Kwao extract (10 mg/ml) was 515.15 \pm 11.11 pg/ml, and ethinylestradiol-17 β (0.52 μ g/ml) was 529 \pm 40.68 pg/ml.

This experiment was carried out to evaluate the influence of White Kwao extract on *in vitro* development of pre-implantation mouse embryo. Two-cells stage were collected and transferred to a petri dish containing 50 μ l of M16 as the control group, M16 containing ethinylestradiol-17 β as the positive control, and M16 containing White Kwao extract. At the 4-cells stage, the embryos cultured in the White Kwao treatment group was significantly different ($p < 0.05$) compared with the control group (95.05% versus 88.34%) and did not significantly different from the positive control group (95.05% versus 93.70%). At 8-cells stage, the embryo cultured in the White Kwao treatment group were not significantly different compared with the control and the positive control group (85.60% versus 78.50% and 82.90%)

When the embryos developed into the morula stage, the embryos culture in the White Kwao treatment group were significantly different ($p < 0.05$) compared with the control group (80.63% versus 69.06%) and were not significantly different from the positive control (80.63% versus 73.00%)

The incubation for the early blastocyst, the embryos cultured in the White Kwao treatment group were significantly ($p < 0.05$) different compared with the control group (72.52% versus 50.22%), and were not significantly different from the positive control group (72.52% versus 63.06%)

The blastocyst formation, expansion and hatching occurred in success during 5 days of incubation. The embryos cultured in the White Kwao treatment group were significantly ($p < 0.05$) differently from the control group (64.41% versus 42.34%), and were not significantly different from the positive control group (64.41% versus 56.31%)

The hatched blastocyst was stained with propidium iodide (PI) and bisbenzimidazole. The numbers of trophectoderm and inner cell mass cells of individual blastocysts were counted by differently labeling the nuclei with two polynucleotide-specific fluorochromes. This experiment was shown that the average number of trophectoderm cells in the blastocysts of the White Kwao treatment group were not significantly different from the control group (80.00 versus 70.00), and the positive control group (80.00 versus 112.50). The average number of inner cell mass in the White Kwao treatment group was significantly different from the control (20.50 versus 20.50), and the positive control group (20.50 versus 16.00)

The other result, trophectoderm (TE) and inner cell mass (ICM) could not be differentiated by this stain. The number of TE and ICM cells of individual blastocysts shown that the total cells of the White Kwao treated blastocyst were not significantly different from the control group (94.25 \pm 9.50 versus 92.33 \pm 4.05), and the positive control (94.25 \pm 9.50 versus 110.33 \pm 9.16).

V. DISCUSSION

The previous studies indicated that estrogen could regulate the cell growth and differentiation by stimulation the local expression of peptide growth factors, which act in an autocrine and/or paracrine mechanism [13]. The recent study in pig reported estrogenic substances called phytoestrogen that found in White Kwao might be playing a role in the embryonic development and gene expression. It is known that growth factors work with the second messenger system, such as cyclic adenosine monophosphate (cAMP) [14].

The present study indicated that the embryos cultivated in the White Kwao treatment group were significantly developed ($p < 0.05$) better than the control at 4-cells, morula, early blastocyst, and late blastocyst stages.

In this study the embryos developed to blastocyst in M16 medium containing White Kwao better than in the control medium. This result supports the estrogenic effect of White Kwao.

Walter and Wheeler [15] explained that estrogen could regulate embryonic cell growth and development via growth factor production. Growth factors such as insulin-like growth factor-I and II (IGF-I and-II) have been shown to increase blastocyst cell number specifically action on the inner cell mass while Colony-stimulating factor-I (CSF-I) is increasing trophectoderm cell number [16].

The experiment was shown that the total cell number and the average of trophectoderm and inner cell mass of blastocyst in the White Kwao treatment group was not significantly different from the control and the positive control group. It might have decreased nutrient in the medium and increased waste product during embryos developing.

However, the embryos cultured in the White Kwao treatment group, and the positive control group exhibited an increase in cell number compared with the control group although it was statistically not significantly ($p < 0.05$)

VI. SUGGESTIONS

In conclusion, the present study demonstrated that the White Kwao has the effect on *in vitro* development of mouse embryos. The exact mechanisms that White Kwao stimulated mouse embryo development were unknown. The suspect mechanism may in a manner similar to the mechanism that estrogen affects the mouse embryo development.

VII. RECOMMENDATION FOR FUTURE RESEARCH

The future studies are needed to transfer the blastocyst into the endometrium of pseudopregnancy mice to evaluate the implantation rate. If the implantation rate is not difference

from the control, and the embryos develop to term with normal phenotype, White Kwao extract can be used instead of estrogen. Since the hormone estrogen is expensive and it has to be purchased from abroad. We can save amount of budget if we perform the research in the fields of *in vitro* development and cloning.

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