CHAPTER 4

DISSCUSSION

A. Effect of White Kwao extract on *in vitro* development of preimplantation mouse embryos.

Embryo development begins at fertilization, the process which male gamete or sperm units with a female gamete or oocyte to form a single cell called zygote. The unicellular organism or zygote divides many times and becomes progressively transformed into a multicellular embryo, migration, growth, and differentiation. The zygote divides into two blastomeres, which then divide into four blastomeres, eight blastomeres, and so on. Cleavage consists of repeated mitotic division of zygote, resulting in a rapid increase in the number of cells. During cleavage, the zygote is within the rather thick, jelly-like zona pellucida that is translucent under the light microscope. Division of the zygote into blastomere begins about 30 hours after fertilization (Sakkas and Vassalli, 2002).

The preimplantation embryo is unique so that it can develop in the absence of direct cell contact with the reproductive tract before implanting in the uterus. The recent study in pig reported that one of the most important factors with maternal recognition of pregnancy is the production of estrogen by the embryo (Walter and Wheeler, 2000). However, the mechanism for estrogen production by the embryo is

unknown. It is not known whether the embryo produces the estrogen or converts another substance into estrogen.

During the developmental phase, the embryo is dependent on the luminal secretions of oviduct and the uterus for its nutrition. Its cellular activities, including cell division, gene expression and metabolism are influenced by the environmental factors, including growth factors, produced by the cells of the reproductive tract. The development of blastocyst *in vivo,* implantation and the maintenance of pregnancy require an effective maternal-embryonic dialogue mediated by growth factors (Tagma, 1992; Hardy and Spanos, 2002).

The mouse or human embryo is relatively self sufficient, can survive and growth in isolation to the blastocyst stage in vitro, in a simple salt solution supplemented only with pyruvate and albumin (Whitten and Biggers, 1968, Devreker et al., 1998).

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 (95.05% vs 88.34%, 80.63% vs 69.06%, 72.52% vs 50.22%, and 64.41% vs 42.34% respectively).

The previous studies indicated that steroid hormone such as estrogen can regulate the cell growth and differentiation by stimulating the local expression of peptide growth factor and their receptors, which act in an autocrine and/or paracrine mechanism (Murphy et al., 1987; Dickson and Lippman, 1987). The role of growth factor in development has been supported by studies in mouse and other species showing that a range of peptide growth factor are produced by the reproductive tract

and preimplantation embryo, while many of their receptors can be detected on the embryo surface. These include members of insulin and insulin-like growth factor (IGF) family, the epideramal growth factor (EGF) family, The fibroblast growth factor (FGF) family, the platelet derived growth factor (PDGF) family and tumor necrosis factor (TNF) family (Kane et al., 1997).

Early studies measured secretion of growth factors by human embryos into the culture medium. Subsequently, expression of mRNA and protein for a variety of growth factor and their receptors have been investigated in single human embryos using sensitive techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR), *in situ* hybridization and immunohistochemistry. Supplementation of culture medium with TGF-α, platelet activating factor (PAF) or IGF-I decreases apoptosis in mouse and rabbit blastocysts *in vitro*, indicating that these growth factor can act as survival factors during preimplantation (O' Neil, 1998, Herrler et al., 1998). Thus, studies in a variety of mammalian species have clearly demonstrated that growth factors are important in blastocyst development and implantation.

It is known that growth factors work with the second messenger system, such as cyclic adenosine monophosphate (cAMP), phosphoinositol or calcium effector pathway (Hill, 1991). There are intracellular mechanisms involved with growth and maturation of tissue classically controlled by hormone such as estrogen (Simmen et al., 1988).

There are many estrogenic substances called phytoestrogen found in the White Kwao such as deoxymiroesterol, daidzein, etc. The recent study reported that the isoflavones might play a role in the embryonic development and gene expression

of early embryos (Walter and Wheeler, 2000). Gene expression of the early embryo may be important by allow certain growth factors to be turned on or off to maintain embryo survival. Effect on protein synthesis, endocytosis, glucose transport, metabolism, gene expression and apoptosis have also been reported in the mouse. Supplementation of culture medium with transforming growth factor α (TGF-α), platelet activating factor (PAF) or insulin-like growth factor-I decrease apoptosis in mouse and rabbit blastocyst *in vitro*, indicating that these growth factors can act as survival factor during preimplantation development (Brison and Schultz, 1977; O'Neil, 1998; Herrler et al., 1998; Brison, 2000). It has been reported that estrogen is required for the transition from the compacted morula stage to the blastocyst (Neimann and Elsaesser, 1986). 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.

B Effect of White Kwao on the number of cells allocate into the inner cell mass (ICM) and trophectoderm (TE) of individual blastocyst

This experiment has the results in two ways. The numbers of trophectoderm (TE) and inner cell mass (ICM) cells of individual blastocysts were counted by differentially labeling the nuclei with two polynucleotide-specific fluorochromes. This experiment was shown that the number average 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 (80.00 versus 112.50). The average number of inner cell mass in the White Kwao treatment group were not significantly different from the control (20.50 versus 20.50), and the positive control group (20.50 versus 16.00).

The other result was shown that the total number of cells of blastocyst in the White Kwao treatment group was not significantly different from the control (94.25 $\pm$ 19.00 vs 92.33 $\pm$ 9.93), and the positive control groups (94.25 $\pm$ 19.00 vs 110.33 $\pm$ 22.43). Using immunosurgery to partially lyse the trophectoderm, it is possible to differentially label the trophectoderm and inner cell mass nuclei of individual fixed blastocyst *in situ*, with two polynucleotide-specific fluorochromes (Handyside and Hunter, 1984). The nuclei can then be distinguished in the basis of color of the fluorescence emission using appropriate filters. The recent study indicated that hormones such as estrogen could regulate embryonic cell growth and development via growth factor production (Walter and Wheeler, 2000). Growth factors have been shown to increase blastocyst cell number, such as, insulin, insulin-like growth factor-I and II (IGF-I and II) specifically action on the inner cell mass while Colony-stimulating factor-1 (CSF-1) is increasing trophectoderm cell number (Harvey and Kaye, 1990, Harvey and Kaye, 1992a, Harvey and Kaye, 1992b, Bhatnagar et al., 1995). A previous study reported that treatment of mouse embryos with PAF increasing blastocyst cell number (Ryan et al., 1996b). In the other cell types, variety growth factors are known to be important in regulation of cell division and cell death. It was therefore logical first to investigate their effects during preimplantation

embryogenesis in laboratory and domestic species, before extending these studies to human embryos. The effect of growth factors can be analysed in a number of ways, including assessing the proportion of embryos developing to the blastocyst stage, rate of development, metabolism, cell number in the blastocyst, and incidence of cell division and cell death.

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 significant ($p<0.05$).

This result revealed that White Kwao extract might have the same effect on embryo development as estrogen

In conclusion, the present study demonstrated that the White Kwao has the effect on the *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. Further 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, the 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.