# CHAPTER 3

# RESULTS

## A. Electro-chemiluminescent immunoassay for estradiol level

The electro-chemiluminescent immunoassay had been established to measure the estradiol level in M16 media containing lyophilized White Kwao extract at 10.00 mg/ml, and the others containing ethinylestradiol-17β 0.52μg/ml before the using of media in embryo culture. This analysis was repeated three times respectively. The results showed that the average level of estradiol value of M16 medium contained lyophilized White Kwao extract (10 mg/ml) was 515.15 ± 11.11 pg/ml, and ethinylestradiol-17β (0.52 μg/ml) was 529.21 ± 40.68 pg/ml. A summary of the results is shown in table 1.

**Table 1. Showing the estradiol values of M16 medium containing White Kwao extract (10 mg/ml) and ethinylestradiol -17β (0.52 μg/ml)**

| No. of test | Estradiol values in M16 containing lyophilized WK extract (10 mg/ml) (pg/ml) | Estradiol values in M16 containing ethinylestradiol (0.52μg/ml) (pg/ml) |
|---|---|---|
| 1 | 515.65 | 561.75 |
| 2 | 526.00 | 483.60 |
| 3 | 503.80 | 542.30 |
| Mean ± SD | 515.15 ± 11.11 | 529.21 ±40.68 |

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

This experiment was carried out to evaluate the influence of White Kwao extract on *in vitro* development of preimplantation mouse embryos. Two-cells stage were collected and transferred to a petri dish containing 50 µl of M16 as the control group, M16 containing ethinylestradiol-17β as the positive control, and M16 containing White Kwao extract (Table2). The number of embryos that developed to hatch blastocysts stage was recorded in every 24 hours (Table3). At 4-cells stage (figure 14), 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, (figure 15), The embryos cultured in the White Kwao treatment group were not significantly different compared with the control and the positive control groups (85.60% versus 78.50%and 82.90%)

When the embryos developed into the morula stage, (figure 16), the embryos cultured 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, (figure 17), 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 blastocysts formation, (figure 18), 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%).

## C. Differential labeling of inner cell mass and trophectoderm

The hatched blastocyst was stained with propidium iodide (PI) and bisbenzimide. The trophectoderm appears pink or red and the inner cell mass is blue. The numbers of trophectoderm (TE) and inner cell mass (ICM) cells of individual blastocysts were counted by differently labeling the nuclei with two polynucleotide-specific fluorochromes (figure 20). 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 not significantly different from the control (20.50 versus 20.50), and the positive control group (20.50 versus 16.00) (Table 3).

The other one, trophectoderm (TE) and inner cell mass (ICM) could not be differentiated by this stain (figure 19). 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 ($94.25\pm9.50$ versus $92.33\pm4.05$), and the positive control ($94.25\pm9.50$ versus $110.33\pm9.16$) (Table4).

**Table 2. Development of mouse embryos from two-cells to blastocyst in three different media (experiment 1-10)**

Experiment 1

| Treatment group | 2-cells | 4-cells | 8-cells | Morula | Early Blastocyst | Late Blastocyst |
|---|---|---|---|---|---|---|
| Control | 34 | 30 | 27 | 15 | 13 | 11 |
| Positive control | 34 | 29 | 29 | 21 | 17 | 15 |
| White Kwao | 34 | 32 | 26 | 23 | 22 | 21 |

Experiment 2

| Treatment group | 2-cells | 4-cells | 8-cells | Morula | Early Blastocyst | Late Blastocyst |
|---|---|---|---|---|---|---|
| Control | 29 | 28 | 26 | 21 | 13 | 7 |
| Positive control | 28 | 25 | 19 | 19 | 13 | 11 |
| White Kwao | 28 | 26 | 21 | 21 | 15 | 14 |

Experiment 3

| Treatment group | 2-cells | 4-cells | 8-cells | Morula | Early Blastocyst | Late Blastocyst |
|---|---|---|---|---|---|---|
| Control | 20 | 17 | 12 | 10 | 9 | 9 |
| Positive control | 20 | 20 | 16 | 15 | 15 | 12 |
| White Kwao | 20 | 19 | 18 | 15 | 15 | 13 |

Experiment 4

| Treatment group | 2-cells | 4-cells | 8-cells | Morula | Early Blastocyst | Late Blastocyst |
|---|---|---|---|---|---|---|
| Control | 20 | 19 | 19 | 13 | 11 | 11 |
| Positive control | 20 | 19 | 17 | 15 | 12 | 10 |
| White Kwao | 20 | 20 | 19 | 19 | 17 | 16 |

Experiment 5

| Treatment group | 2-cells | 4-cells | 8-cells | Morula | Early Blastocyst | Late Blastocyst |
|---|---|---|---|---|---|---|
| Control | 20 | 19 | 16 | 13 | 12 | 11 |
| Positive control | 20 | 19 | 17 | 14 | 15 | 15 |
| White Kwao | 20 | 20 | 20 | 18 | 17 | 16 |

Experiment 6

| Treatment group | 2-cells | 4-cells | 8-cells | Morula | Early Blastocyst | Late Blastocyst |
|---|---|---|---|---|---|---|
| Control | 20 | 17 | 13 | 10 | 8 | 7 |
| Positive control | 20 | 19 | 16 | 15 | 12 | 12 |
| White Kwao | 20 | 18 | 16 | 15 | 14 | 12 |

Experiment 7

| Treatment group | 2-cells | 4-cells | 8-cells | Morula | Early Blastocyst | Late Blastocyst |
|---|---|---|---|---|---|---|
| Control | 20 | 17 | 13 | 12 | 9 | 8 |
| Positive control | 20 | 19 | 15 | 14 | 11 | 11 |
| White Kwao | 20 | 19 | 16 | 15 | 14 | 13 |

Experiment 8

| Treatment group | 2-cells | 4-cells | 8-cells | Morula | Early Blastocyst | Late Blastocyst |
|---|---|---|---|---|---|---|
| Control | 20 | 15 | 14 | 9 | 9 | 9 |
| Positive control | 20 | 19 | 17 | 14 | 14 | 12 |
| White Kwao | 20 | 18 | 17 | 16 | 13 | 13 |

Experiment 9

| Treatment group | 2-cells | 4-cells | 8-cells | Morula | Early Blastocyst | Late Blastocyst |
|---|---|---|---|---|---|---|
| Control | 20 | 17 | 17 | 16 | 13 | 11 |
| Positive control | 20 | 19 | 18 | 17 | 17 | 17 |
| White Kwao | 20 | 19 | 19 | 19 | 16 | 16 |

Experiment 10

| Treatment group | Total of 2-cells | 4-cells | 8-cells | Morula | Early Blastocyst | Late Blastocyst |
|---|---|---|---|---|---|---|
| Control | 20 | 18 | 18 | 15 | 15 | 13 |
| Positive control | 20 | 20 | 20 | 18 | 14 | 14 |
| White Kwao | 20 | 20 | 18 | 18 | 18 | 18 |

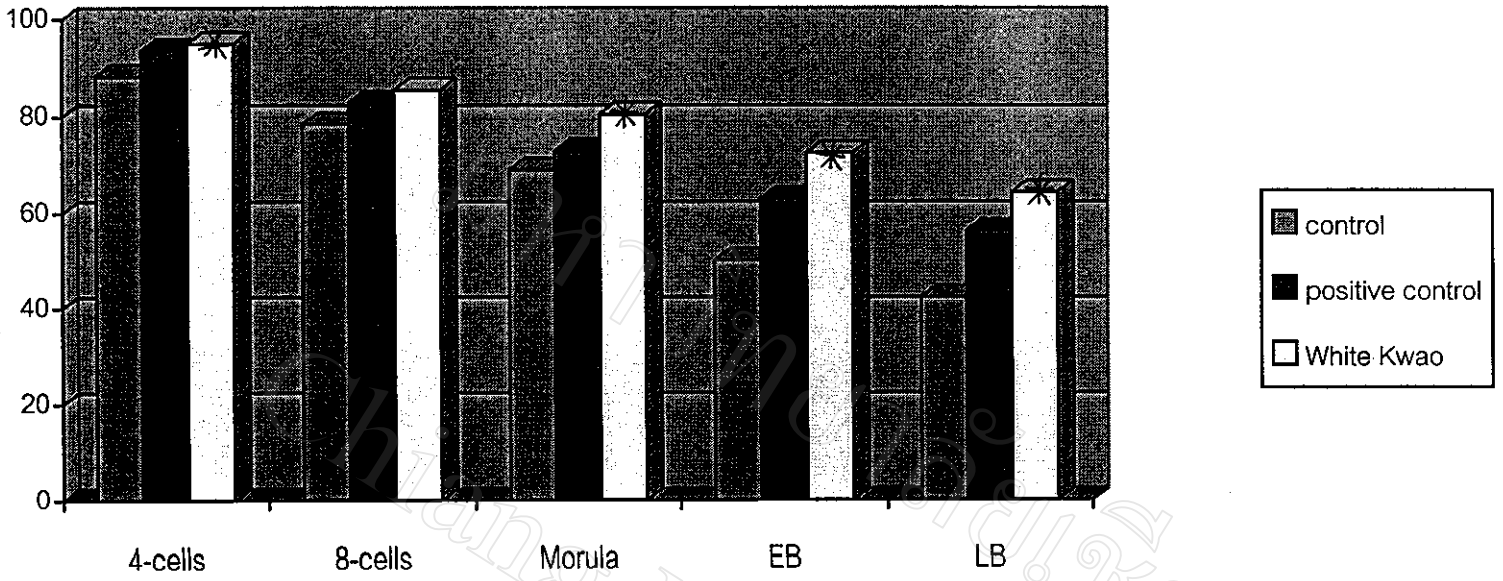
## D. Effect of White Kwao extract on implantation rate of mouse blastocysts

The implantation rate of mouse embryos was also planned to evaluate. The experimental mice were stimulated for superovulation and they were mated with male mice. After fertilization for 40-44 hours, the two-cells stage were collected (figure 13). The two-cell stage embryos were then cultivated in three difference media. The embryos were all failed to develope to the desired stage because of the inappropriate incubator adjustment. The result of this part was incomplete.

**Table 3 The percentage of mouse embryos developed from two-cells stage to blastocyst after they were grown in the three difference media**

| Treatment group | Total of 2-cells | 4-cells (%) | 8-cells (%) | Morula (%) | Early Blastocyst (%) | Late Blastocyst (%) |
|---|---|---|---|---|---|---|
| CONTROL | 223 | 197(88.34) | 175(78.50) | 134(69.06) | 112(50.22) | 94(42.34) |
| POSITIVE-CONTROL | 222 | 208(93.70) | 184(82.90) | 162(73.00) | 140(63.06) | 125(56.31) |
| WHITE KWAO | 222 | 211(95.05)* | 190(85.60) | 179(80.63)* | 161(72.52)* | 143(64.41)* |

*indicates significantly ($p<0.05$) different from the control group




* indicates significantly different ($p<0.05$) from the control group

EB: Early blastocyst

LB: Late blastocyst

**Figure 12 Histogram shows the comparison of the development of the mouse embryos from 4-cells stage to late blastocyst, after they were cultivated in the three difference media**

**Table 4 A comparison of the cell number of trophectoderm and inner cell mass after they were cultivated in the three difference media for 5 days**

| Group | Total blastocyst (n) | Trophectoderm cells (mean) | Inner cell mass (mean) |
|---|---|---|---|
| CONTROL | 1 | 70 (70) | 16 (16) |
| POSITIVE CONTROL | 2 | 112.50 (91-134) | 20.50 (18-23) |
| WHITE KWAO | 2 | 80 (77-83) | 20.50 (17-24) |

**Table 5 A comparison of total cell number of blastocyst after they were cultivated in the three differences media for 5 days**

| Group | Total blastocyst (n) | Total cells No. per Blastocyst (Mean ± S.E.M) |
|---|---|---|
| CONTROL | 6 | 92.33 ± 4.05 |
| POSITIVE CONTROL | 6 | 110.33 ± 9.16 |
| WHITE KWAO | 4 | 94.25 ± 9.50 |

**Figure 13 Photomicrograph of representative 2-cells stage(x 100)**

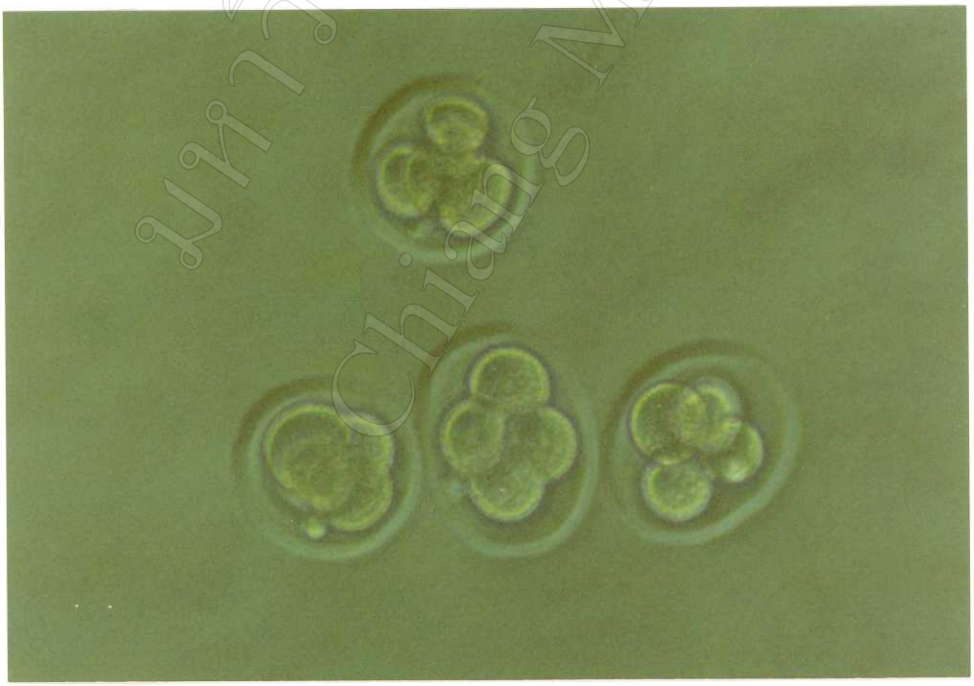


**Figure 14 Photomicrograph of representative 4-cells stage(x 100)**

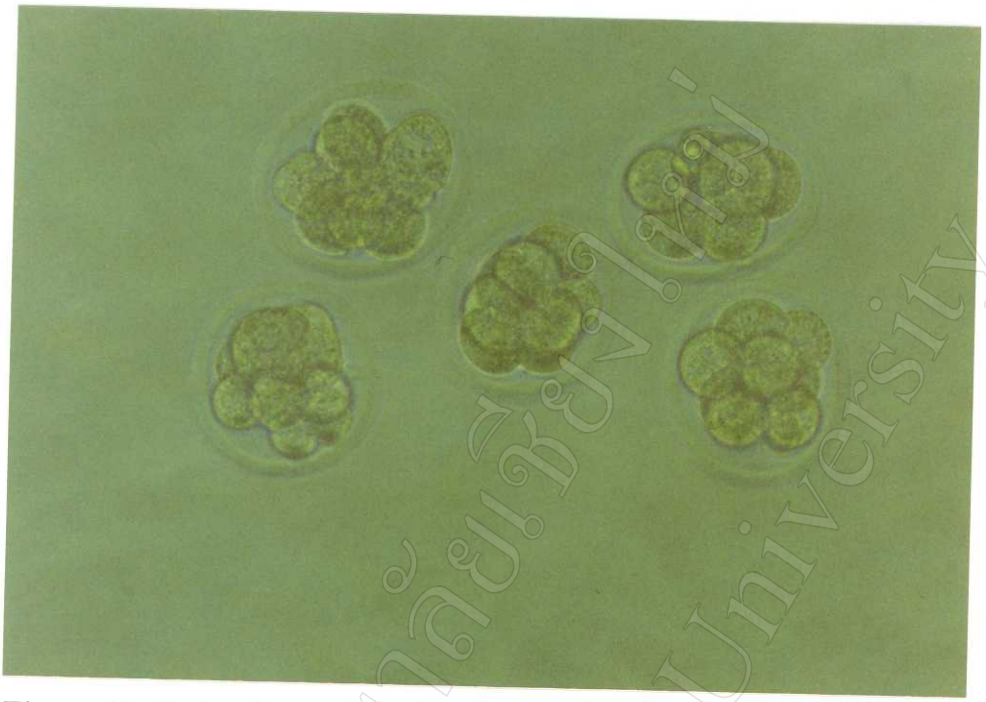


**Figure 15 Photomicrograph of representative 8-cells stage(x 100)**

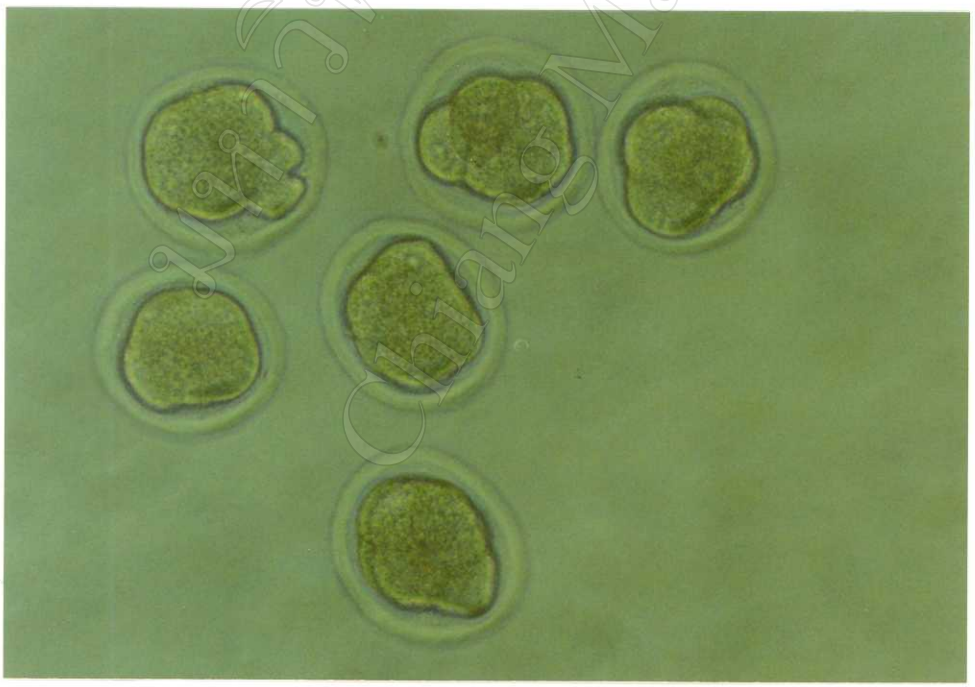


**Figure 16 Photomicrograph of representative morula stage(x 100)**

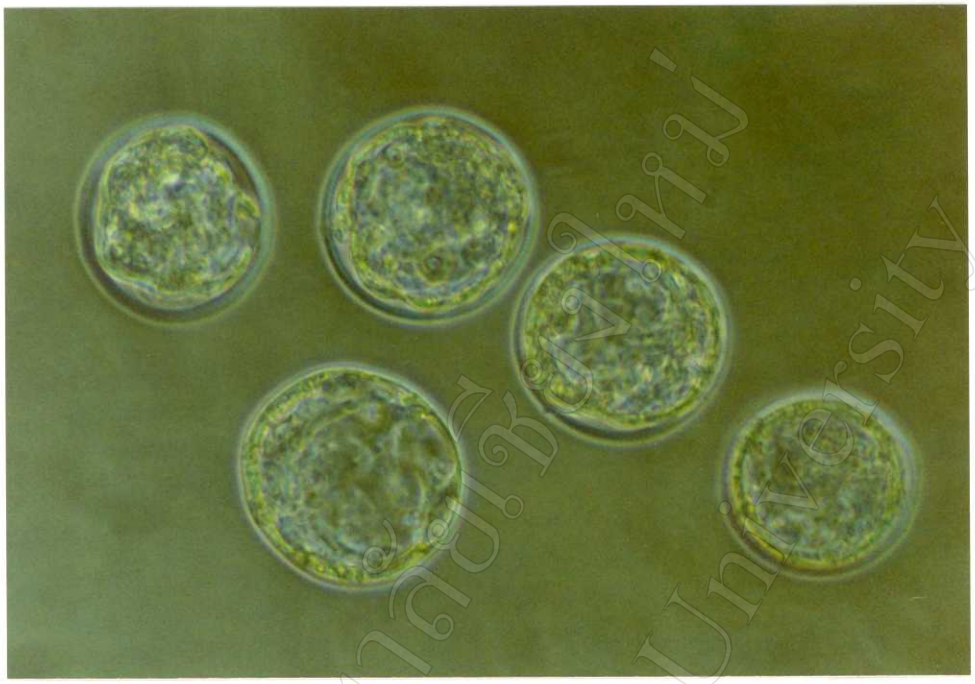


**Figure 17 Photomicrograph of representative late blastocyst stage(x 100)**

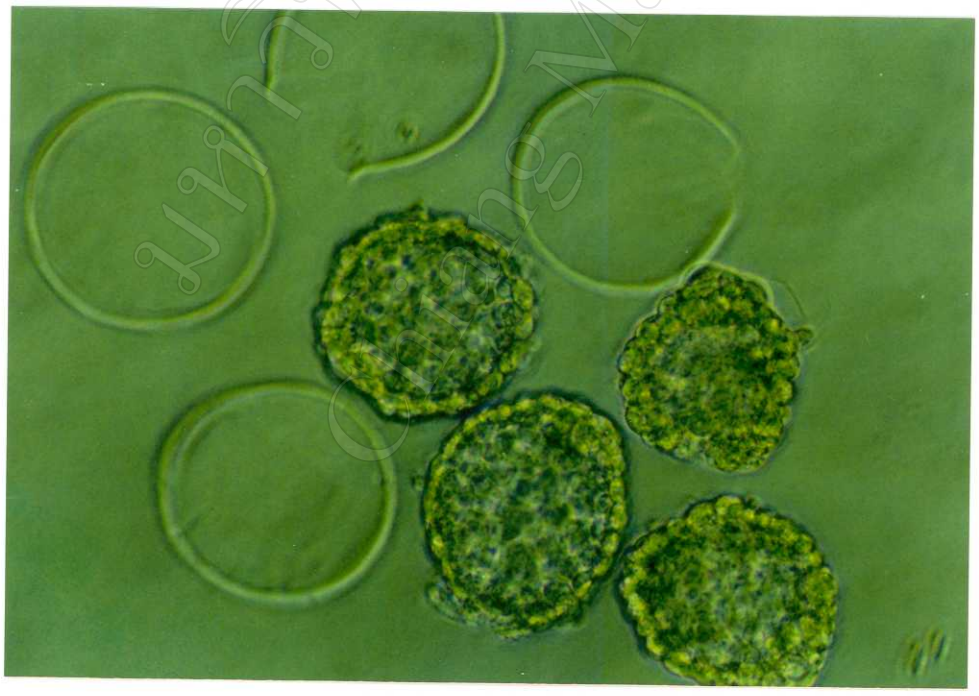


**Figure 18 Photomicrograph of representative hatch blastocyst stage(x 100)**

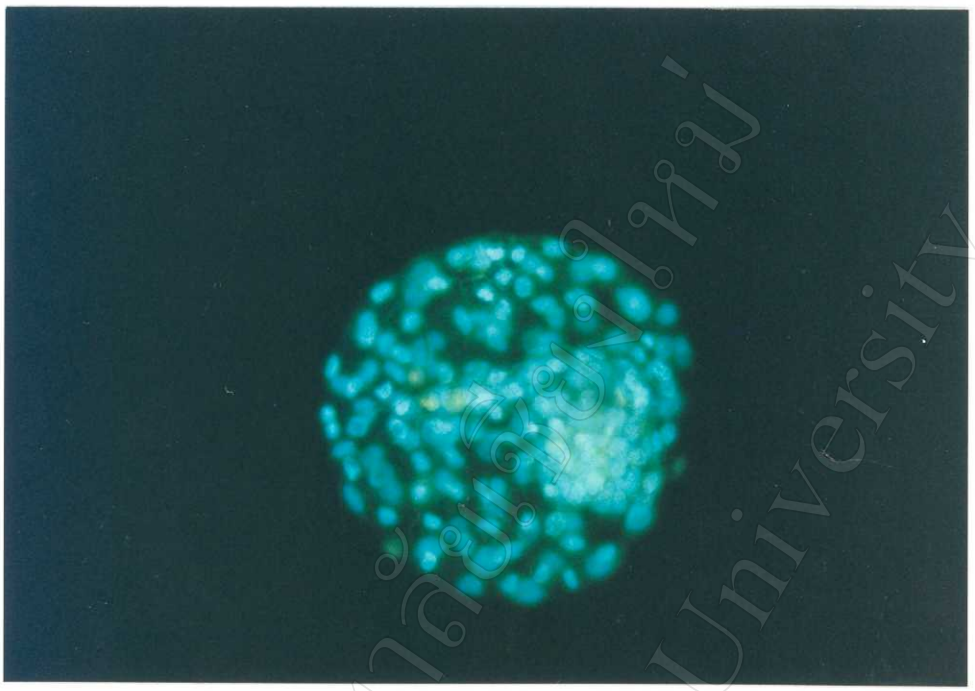


**Figure 19 Photomicrograph of representative blastocyst stained with propidium iodide and bisbenzimde(x 400)**

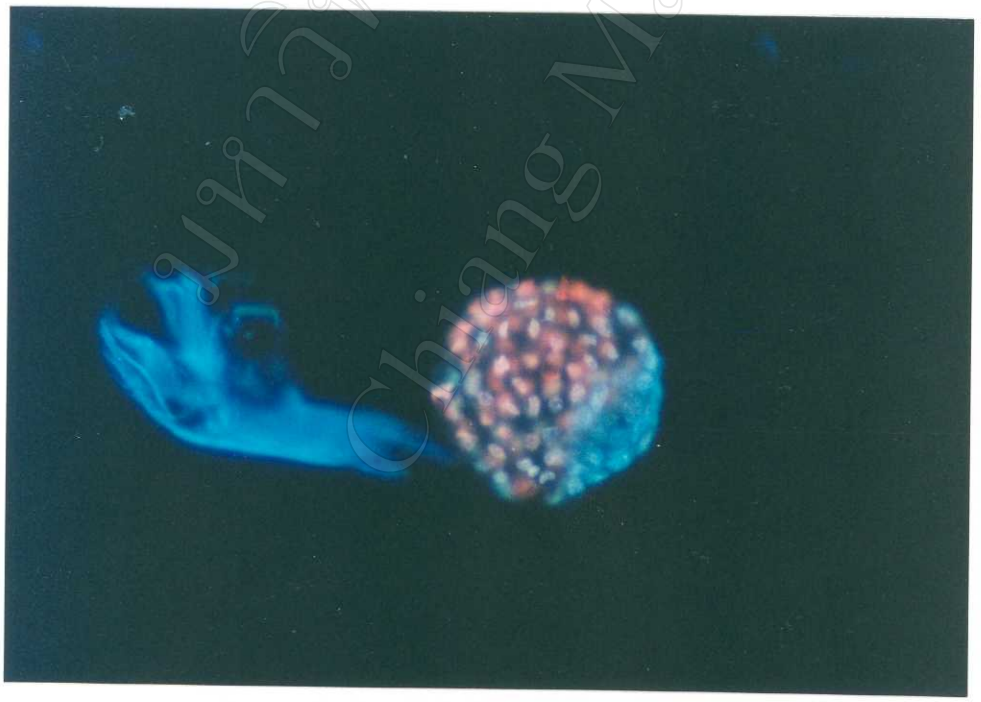


**Figure 20. Photomicrograph of differential stained the inner cell mass (blue) and trophectoderm (red) after they were stained with propidium iodide and bisbenzimide(x 400)**