CHAPTER III

MATERIALS AND METHOD

1. Equipment

1. Laminar Flow hood	: Model GLG-48 Gensa Company limited Bangkok, Thailand.
2. Water Jacket CO_2Incubator	: Model 3030 Forma Scientific, Ohio, USA.
3. Advanced Osmometer	: Model 3 D2 1000 Highland avenue, Massachusetts, USA.
4. pH meter	: Cole Paarmer, Digi pH ase, Siam Medico Supply, Thailand.
5. Inverted microscopic	: Olympus, Japan
6. Dissection Microscope	: Olympus, Japan
7. Balance	: Model JP-160 Chiyo Balance, Corporation, Tokyo, Japan.
8. Plastic tissue culture dish	: Nunclon, Roskildi, Denmark
9. Plastic Syringe 1 ml, 10 ml.	: Terumo Corporation, Tokyo, Japan
10. Embryological watchglass	: HLJ-630-S 4-cm-squre glass block with a 3 mm. diameter cavity Griffin & George Co.
11. Millipore Filter, Plastic Swinney adapter type, and	: Filter type HA. Pore size 0.22 micron

Millipore membrane	Millipore Corporation, Bedford, Massachusetts, USA.
12. Hypodermic needles	: Size G 30x3/4" Stainless steel luer Monts, England.

13. Month pieces
14. Pasteur pipette
15. Curved scissors
16. Straight scissors
17. Watch-Maker forceps
18. Hot air oven
19. Autoclave

2. Chemicals

2.1 Media compositions

2.1.1 Bovine Serum Albumin Fraction V
: Sigma Chemical Company, St. Louis, MO, USA.

2.1.2 Calcium Chloride Dihydrate ($CaCl_2.2H_2O$)
: E.Merck Dermstadt, Germany.

2.1.3 Magnesium Chloride Hexahydrate ($MgCl_2.6\ H_2O$)
: E.Merck Dermstadt, Germany

2.1.4 Potassium Chloride: E.Merck Dermstadt, Germany

2.1.5 Penicillin G. sodium: E.Merck Dermstadt, Germany

2.1.6 Sodium Chloride (NaCl): E.Merck Dermstadt, Germany

2.1.7 Sodium bicarbonate ($NaHCO_3$)
: E.Merck Dermstadt, Germany

2.1.8 Sodium Lactate (Na Lactate 60%Syrup)
: Sigma Chemical Company, St. Louis, MO, USA.

2.1.9 Mineral Oil: Sigma Chemical Company, St. Louis, MO, USA.

2.2 Superovulation Hormone

2.2.1 Gonadotropin (Pregnant Mare's Serum)

: Sigma Chemical Company, St. Louis, MO, USA.

2.3 Amino acids

2.3.1 Asparagine: Sigma Chemical Company, St. Louis, MO, USA.

2.3.2 Aspartate: Sigma Chemical Company, St. Louis, MO, USA.

2.3.3 Cysteine: Sigma Chemical Company, St. Louis, MO, USA.

2.3.4 Glutamate: Sigma Chemical Company, St. Louis, MO, USA.

2.3.5 Glutamine: Sigma Chemical Company, St. Louis, MO, USA.

2.3.6 Glycine: Sigma Chemical Company, St. Louis, MO, USA.

2.3.7 Histidine: Aldrich

2.3.8 Lysine: Sigma Chemical Company, St. Louis, MO, USA.

2.3.9 Proline: Sigma Chemical Company, St. Louis, MO, USA.

2.3.10 Serine: Sigma Chemical Company, St. Louis, MO, USA.

2.3.11 Taurine: Sigma Chemical Company, St. Louis, MO, USA.

2.4 Vitamin

2.4.1 **Pantothenate**: Sigma Chemical Company, St. Louis, MO, USA.

3. Animals

Hamsters were bred and fed on mouse food and clean water in animal house at Department of Biology, Faculty of Science, Chulalongkorn University. They were maintained on 14hr. light: 10hr. Dark lighting schedule (light on at 6:oo a.m.) at 24–26 ^{0}c. Female hamsters between 2 and 6 months of age were checked daily for the estrous cycles by gently squeezing around the vagina. The day a vaginal discharge found was designated as day 1 of the cycle.

4. Superovulation

Female hamsters weighing 90-100 g were injected with 25 IU of PMSG (Sigma) i.p. at 4.00 –5.00 p.m. on day 1 of cycle. In the evening of day 4, female hamsters were placed with the fertility proven male hamsters. In the next morning, females were separated from males and checked for the present of sperms in the vagina to confirm the successful mating, the day was designated as day 1 of pregnancy.

5. Preparation of equipment

Glass, metal, and plastic used in the embryo culture work were cleaned in 2% 7X cleaning solution (Flow Laboratories, USA.). After several rinsed with tap water, final rinsed with triple distilled water. The equipment was dried in a hot air oven at 80 ^{0}c for three hours. The dried items were covered with aluminum foils. Glass and metals were sterilized in the hot air oven at 150 ^{0}c for two hours. Plastics were sterilized by autoclave at 121 ^{0}c at 15 pounds per square inch for thirty minutes.

6. Collection of Embryo

Successfully mated females were killed by cervical dislocation between 9.00-11.00 a.m. on day 2 of pregnancy to collect two–cell embryos, and between 5.00- 7.00 p.m. on the day 3 of pregnancy to collect eight–cell embryos. Oviducts of day 2 pregnant hamsters and uteri of day 3 pregnant hamsters were cut out and laid on a piece of filter papers to remove excess blood. They were transferred to a drop of HECM–10

in a sterile embryological watchglass. Two–cell embryos were flushed from excised oviducts and eight – cell embryos were flushed from excised uteri. Embryos were flushed with 1.0 ml of HECM-10 without amino acids and vitamin. The embryos were picked up with a sterile pulled Pasteur pipette. They were washed twice in HECM–10 and randomly distributed to various culture drops in culture dishes.

7. Culture Media

The basic medium used for culture of embryos was Hamster Embryo Culture Medium- 10 (HECM-10)(Lane *et al.*, 1998). Medium was prepared at 275 mOsmol, 325 mOsmol and 375 mOsmol by adding 113.5 mM NaCL, 126.5 mM NaCl and 152mM NaCl. The compositions of the medium were given in table 1. All chemicals were analytical grade reagent and embryo culture grade. All salts were purchased from Merck. All amino acids, Bovine Serum Albumin (BSA), Lactic acid and vitamin were obtained from Sigma and Aldrich. The concentration of glutamine, glycine, and taurine were 0.2 mM, 0.05 mM, and 0.5 mM. Amino acids and vitamin were prepared as 100x concentration stock solution and frozen at 0 ^{0}c. They were added to basic medium at 10 µl/ml on the day of experiment.

8. Preparation of culture media

Chemicals were divided into two groups. The first group was all chemicals except bicarbonate. Each of chemicals in the first group was dissolved in triple distilled water completely. Bicarbonate was dissolved separately in triple distilled water and it was added to the first solution at last. Medium was equilibrated by bubbling 5% CO_2 in air until the medium color changed from pink to orange. pH was measured by pH meter and adjusted to 7.2 $\pm$ 0.1 by adding 0.1 M NaOH or HCL. The osmolarity was determined by osmometer. Later, BSA (3 mg/ml) was added into medium. Medium was filtered through Millipore membrane (GS, 0.22 µ pore size, Millipore Corp.) The sterile medium was stored at 4 ^{0}c and made up fresh every two weeks.

9. Embryo culture

Culture drops were prepared by following to the microdrop method. Drops containing 50 μl of culture medium were set in 35x10 mm. Nunclon plastic tissue culture dishes under mineral oil (Sigma). There were six drops in a dish. The first drop was the control group and the other drops were experimental groups. The culture dishes were equilibrated for at least 3 hr. in humidified 5%CO_2 incubator at 37 ^{0}c before embryos were cultured. Embryos in each drop ranged from 10 to 20. After embryos were transferred, all culture dishes were maintained in the incubator.

10. Experimental design

This study aims to find out the protective effects of glycine, glutamine and taurine on the development of two-cell and eight–cell hamster embryos in HECM-10 which have abnormally high osmolarity. The osmolarity chosen for normal, high, and extra high were 275 mOsmol, 325 mOsmol and 375 mOsmol, respectively. The concentration of glycine, glutamine and taurine were 0.05, 0.2, and 0.5. The experimental design for embryo culture was as follow:

Table 2 Experimental design for embryo culture in various media and osmolarities

Category	Osmolarity (mOsmol)		
	275	325	375
HECM-10 (control)	*	*	*
HECM-10 + glycine	*	*	*
HECM-10 + glutamine	*	*	*
HECM-10 + taurine	*	*	*
HECM-10 + glycine + glutamine	*	*	*
HECM-10 + glycine + taurine	*	*	*
HECM-10 + glutamine + taurine	*	*	*
HECM-10 + glucine + glutamine + taurine	*	*	*

* Represent group of embryos being cultured

11. Data collection and Statistic

Embryos were examined by inverted microscopic at 24, 48 and 72 hr. The number of embryos that developed to the blastocyst stage was recorded. Developmental data were expressed as a percentage of embryos that reached blastocysts stage over the total number of embryos cultured. Differences among treatments were subjected to two–way analysis of variance (ANOVA) by using the general linear models (GLM) procedure of SPSS. Using Fisher's least significant difference test (LSD) compared treatments. Significance was set at $p<0.05$.

Table 3 Compositions of Hamster Embryo Culture Medium-10 (HECM-10) for hamster embryo culture.

Component	M.W.	mM	mg/100ml
NaCl	58.44	106.5	622.38
KCL	74.56	3.0	22.368
$CaCl_2.2HO$	147.02	1.0	14.702
$MgCl_2.6HO$	203.31	2.0	40.662
$NaHCO_3$	84.01	25.0	210.025
Na-lactate	112.10	3.5	0.05 ml
Asparagine	132.1	0.01	0.1321
Aspartic acid	133.1	0.01	0.1331
Glutamic acid	147.1	0.01	0.1471
Cysteine	121.6	0.01	0.1216
Proline	115.1	0.01	0.1151
Histidine	155.1	0.01	0.1551
Lysine	146.2	0.01	0.1462
Serine	105.1	0.01	0.1051
Pantothenate	238.3	3μM	0.7149
BSA	-	-	3 mg/ml
Penicillin-G	-	-	6.0
Phenol red	-	-	-

pH = 7.2-7.4

osmolarity = 275± 10 mOsmol

(Lane *et al.*, 1998)

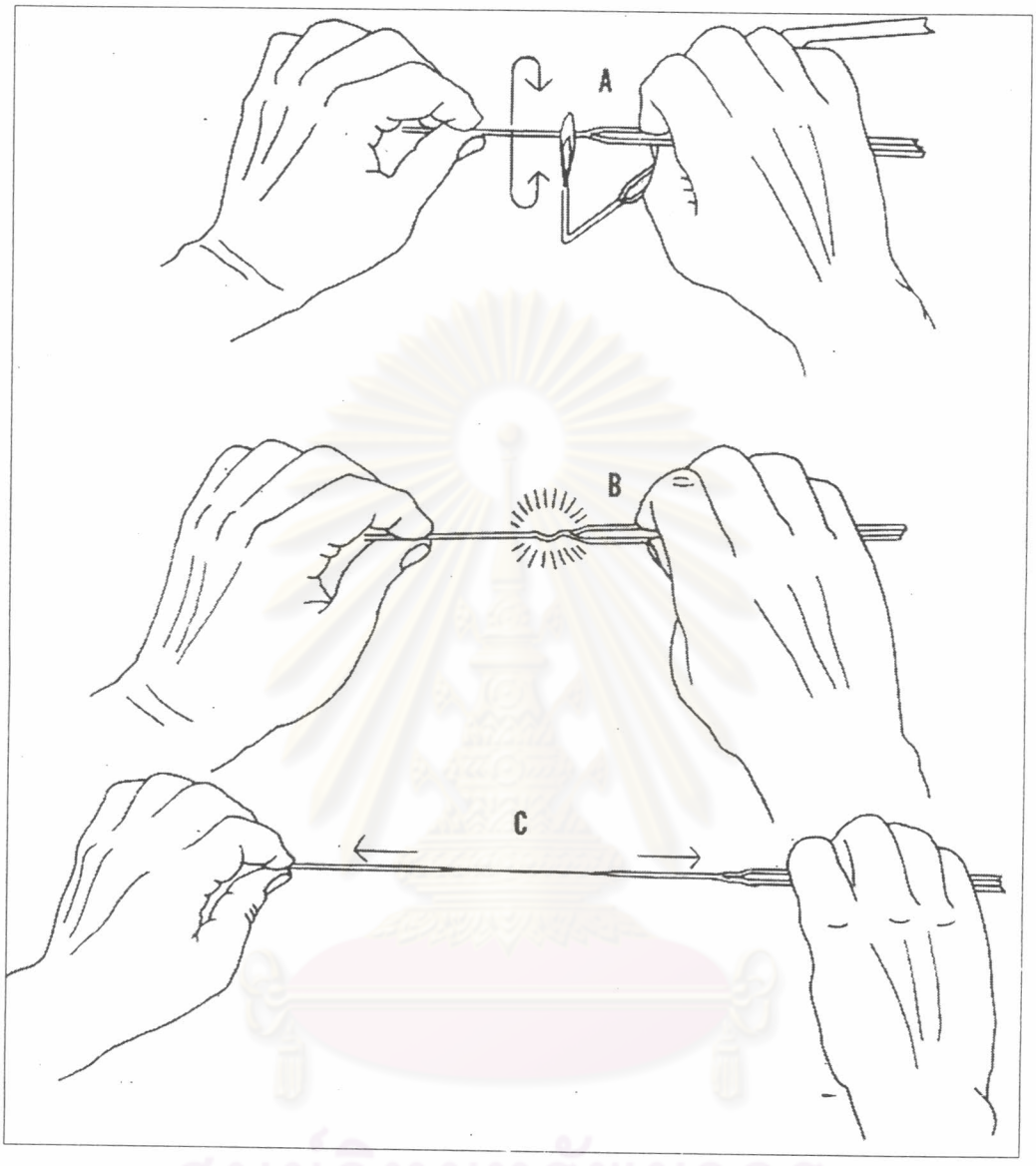


Figure 2 Preparation of micropipettes

(Reproduced from Rafferty, 1970)

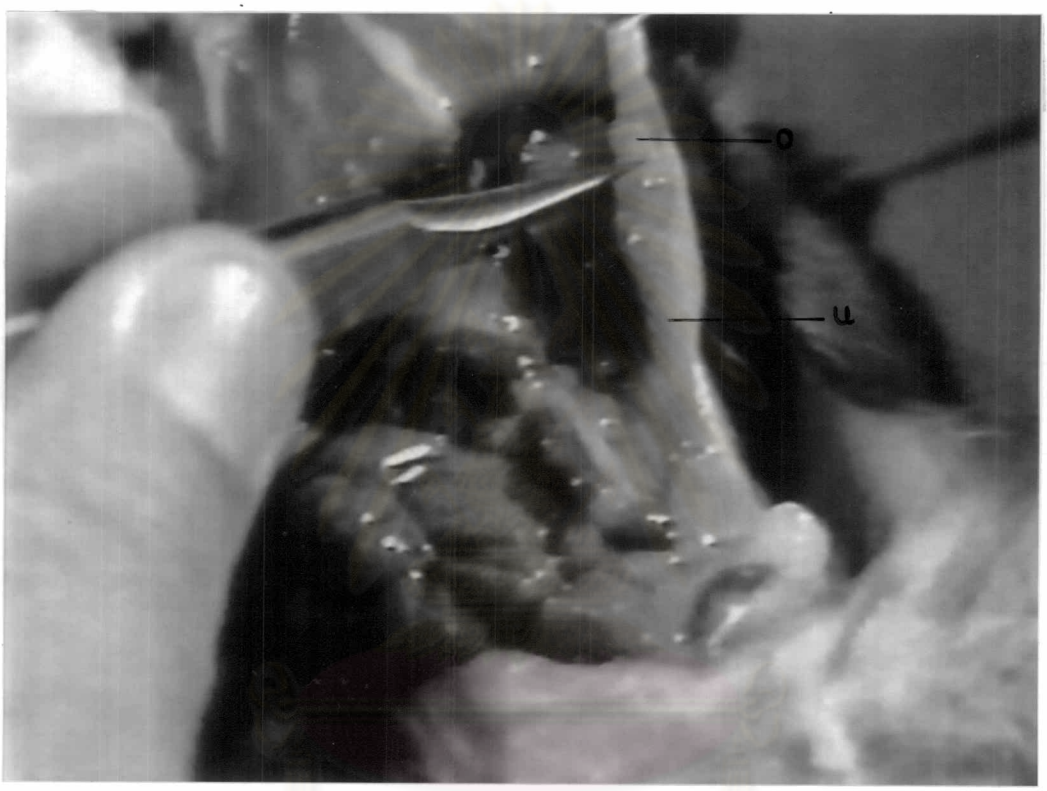
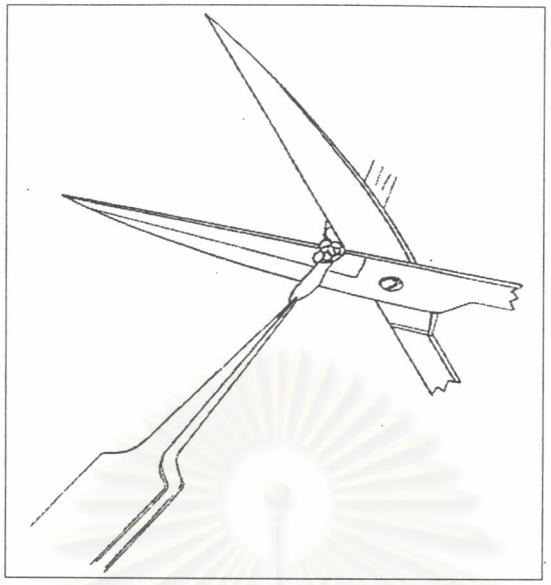


Figure 3 Female hamster reproductive organs: oviduct (o) and uterus (u)

a



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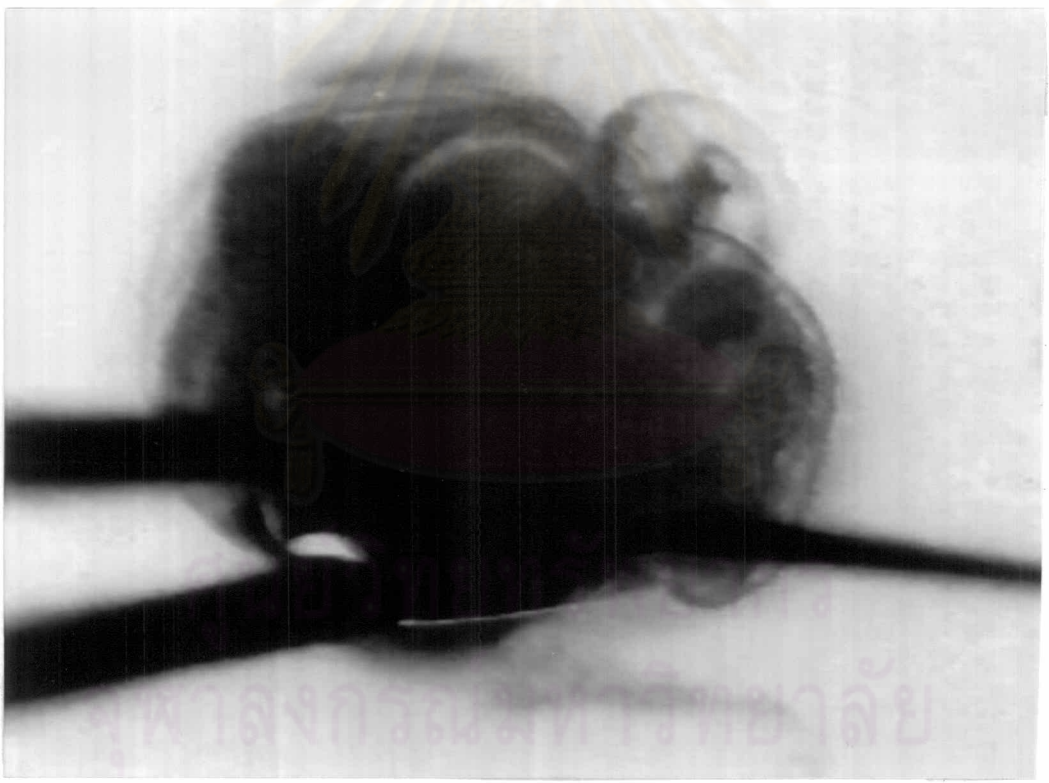

Figure 4 Embryo collection.

(a) Removal of oviduct (Reproduced from Rafferty, 1970)

(b) Oviductal flushing