CHAPTER II

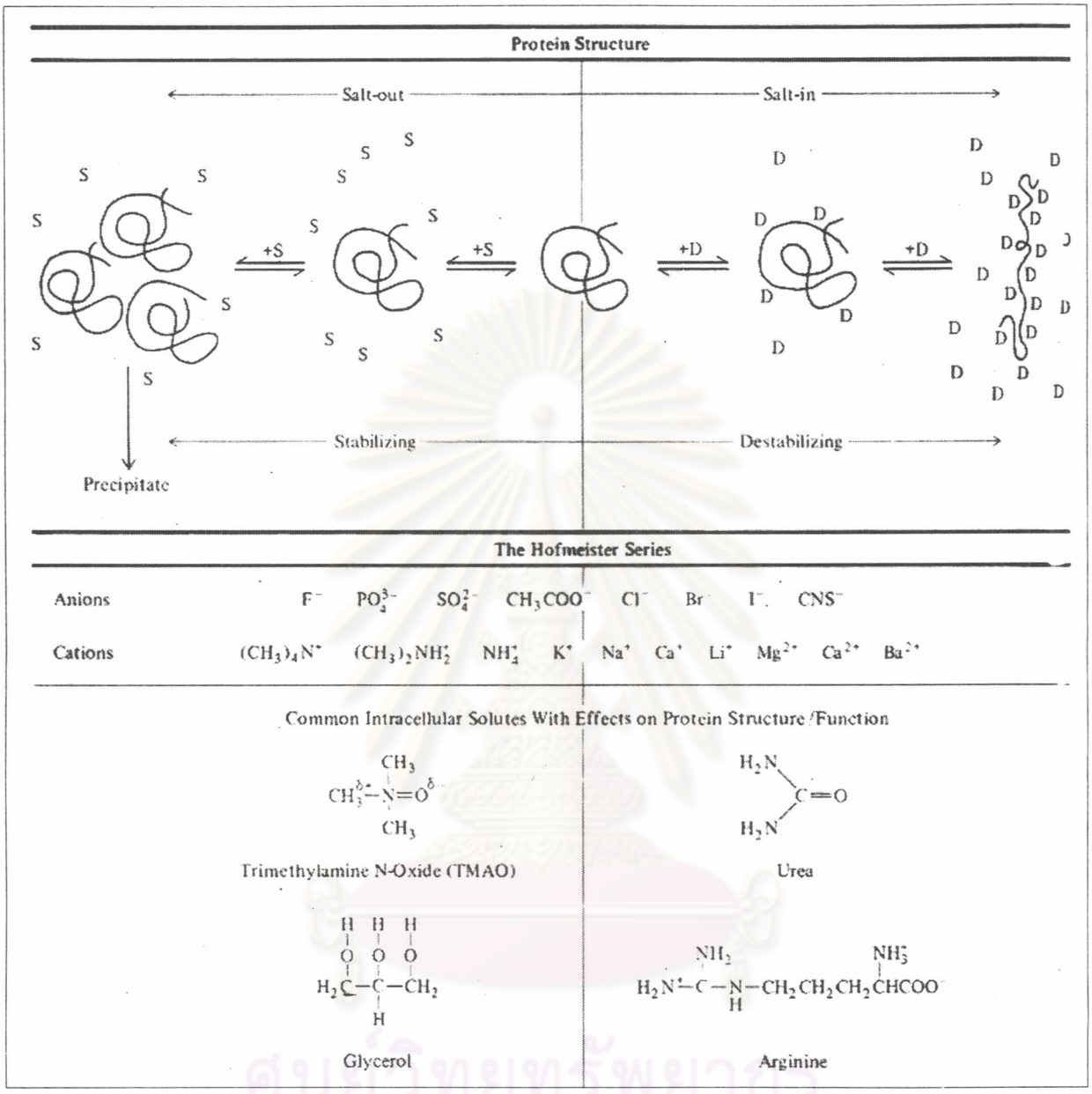
LITERATURE REVIEWS

1.Osmolyte

Cell volume is perturbed from osmotic imbalance environment. If cell is exposed to hypotonic extracellular fluid, they initially swell but then approach the original cell volume by release of solutes through separate K^+, Cl^- and organic osmolytes channels, K^+, Cl^- cotransporters, or coupled K^+, H^+ and Cl^-/HCO_3^- exchangers. This mechanism is called regulatory volume decrease (RVD). In contrast, if cells are exposed to hypertonic extracellular fluid, they initially shrink but then approach the original volume by import of solutes through Na^+, K^+, Cl^- cotransporter as well as Na^+/H^+ and Cl^-/ HCO_3^- exchanger working in parallel. This mechanism is called regulatory volume increase (RVI). (Hallows and Knaut, 1994; Hoffmann and Mills, 1999). The cellular accumulation of electrolytes after cell shrinkage is limited because high ion concentration interfere with structure and function proteins, presumably through ionic interactions with macromolecule and substrate charges. (Greenway and Osmond, 1972; Yancey *et al.*, 1982; Clark, 1987; and Yancey, 1994). Furthermore, attraction of ion gradients across the cell membrane would affect the respective transporters. An increase of intracellular Na^+ activity, for instance, would reverse Na^+/Ca^+ exchange and thus increase intracellular Ca^+ activity, which would affect a multitude of cellular functions (Lang *et al.*, 1998; Trump and Breezesky, 1995).

The organic osmolytes are used for adjusting osmotic pressure without compromising proteins, so they are termed the compatible osmolytes. Unlike ions, compatible osmolytes at high concentration do not compromise the protein functions because their properties are similar to anions and cations of Hofmeister series. Ions on the left of series generally stabilize proteins, salt out, while those ions to the right do the opposite (destabilize protein) (shown in Figure 1). The effects from destabilizing ion such as K^+, Na^+, and urea can be counteracted by adding the stabilizing ions that is so-

called the additive algebraically effect. The destabilizing organic may be attracted to specific chemical groups on macromolecules. Alternatively, they may bind to water less well than water does to itself, so those solutes move away from water and towards the surfaces of macromolecules. If either effect dominates then macromolecules will unfold, since this will maximize these favorable interactions. In contrast, organic stabilizers exhibit an empirical tendency to be excluded from the protein hydration domain shell. If this effect dominates, an entropically unfavorable order arises with neighboring regions of high and low solute. Proteins reduce this order if they minimize their exposed surface areas, by folding more compactly, by aggregation, or precipitation (salting out); i.e., they will be stabilized (Na and Timasheff, 1981; Yancey *et at.*, 1982; Somero, 1986; and Yancey, 1994). Stimulated uptake, enhanced formation, or decrease degradation can increase cellular osmolytes. As compared with RVI accomplished by ions, accumulation of omolytes is a slow process taking hours to days (Kinne, 1993.; Burg, 1994.; Burg, 1995.; Kwon and Handler, 1995.; Lang *et al.,* 1998.; Handler and Kwon, 2001.)



(Reproduced from Withers, 1992)

Figure 1. Effects of a stabilizing solute (S) and a destabilizing solute (D)

The osmolytes are divided into three groups. There are small zwitterionic amino acids such as taurine, glutamate, and glutamine, methylammonium compounds such as betaine and the last group is sugars and polyols such as glucose and inositol.(Yancey, 1994). Organic osmolytes are involed in cell volume regulation in many kinds of organisms. Some aquatic animals can adjust their osmotic concentration to equal to the external environment such as hagfish, but all marine elasmobranchs regulate their total osmotic concentration up to that of seawater while their internal salt concentration equal to about one–third that of seawater. The reason is that they accumulate large quantities of urea and organic osmolytes (trimethylamine oxide; TMAO) in the typical 2:1 concentration ratios in their blood (Moyle and Cech, 2000). From the algebraically additive effect, TMAO protects the elasmobrach enzymes from the perturbing effects of urea (Wither, 1992). Because of the formation of concentrated urine, the renal medulla becomes hypotonic in association. The renal medulla cells response this stress by accumulating organic osmolytes. The studies on culture kidney–derived cells and intact cells in the hypertonic medium show that the renal medulla osmolytes are sorbitol, inositol, betaine, and glycerophosrylcholine (GPC) (Bangnasco *et al.*, 1986; Garcia-Perez and Burg, 1991a; Garcia –Perez and Burg, 1991b; Law, 1991; Handler and Kwon, 1993; Miller, Hanson, and Yancey, 2000).

Like the other organisms, which face the osmotic stress, many bacteria accumulate osmolytes. For example, *Dunaliela tertiolecta* and *Dunuliella viridis* accumulate glycerol (Borowitzka *et al.*, 1977). *Methanogenium caricai* and *Methanococcus thermolithotrophicus* use L-alpha- glutamate, beta-glutamate, N epsilon-acetyl-beta lysine and betaine to maintain their osmotic pressure (Miller and Wood, 1996). Ectine functions as an osmolyte in the moderate halophile, *Halomonas elongata* OUT30018. Nakayama *et al.* (2000) transferred three genes encoding the enzyme involved in the biosynthesis of ectoine into cultured tobacco (*Nicotiana tabacum L.*) CV Bright Yellow 2 (BY2) cells. The transgenic BY2 cells accumulated ectoine and

exhibited a normal growth pattern under hyperosmotic conditions (up to 530 mOsmol) compared with wild type cells. The distribution of osmolytes is compiled in Table 1.

Table 1 Distributions of Organic Osmolytes in Major Organism Groups

Organism	Sugars and polyols	Amino acid, Derivatives	Methylated Ammonium, Sulfonium Solutes
Archebacteria: Methanogens		Glutamine, glutamate Proline, β-glutamate, β-glutamine, N^{ε}- acetyl-β-lysine	Glycine betaine
Eubacteria	Trehalose, surcose, mannitol, heteroside (glycosylglycerol), mannosucrose	Glutamine, glutamate proline, ectoine, hydroxyectoine, N^{ε}-carbomoyl-L-glutamate 1-amide, *N*-acetylglutaminyl-glutamine amide, pipecolate, GABA	Glycine betaine, choline –*O*-sulfate, proline betaine, taurine betaine, β-alanine betaine, pipcolate betaine, glutamate betaine, DMSP
Protista: Algae	Glycerol, mannitol, sorbitol, volemitol, altritol, surcose, mannose, glucose, heterosides (e.g. floridoside), cyclohexane	Proline, alanine, glycine, glutamate	Glycine betaine, proline betaine, homarine, choline-*O*-sulfate, DMSP, dimethyl-taurine, tauine betaine
Protozoa		Glycine, alaine, proline	
Yeast, Fungi	Glycerol, erythriol, arabitol, mannitol, etc.		
Vascular plants	Myo-inositol, methyl-inositol(e.g. pintol, onoitol), sorbitol, mannitol, glucose, surcose	Proline	Glycine betaine, proline betine, β-alaline betaine, Choline-O-sulfate, DMSP

Organism	Sugars and Polyols	Amino Acid, Derivatives	Methyllated Aminonium, Sulfonium Solutes
Animals			
Various aquatic	Glycerol, trehalose (*Artemia*)	Proline, serine (insects)	Glycine betaine, TMAO Proline betaine (marine
Invertebrates	Trehalose(insects)	Glycine, alanine, proline, serine, taurine (marine phyla)	phyla),TMAO (*Artemia*)
Agnathans		Glycine, alaine, proline, etc	TMAO
Cartilaginous fishes	*Myo*-inositol, *scyllo*-inositol	Taurine, β-alanine, glycine, alanine, ect.	Glycine betaine, TMAO Sarcosine
Bony fishes	Glycerol (antifreeze)	γ-amino butyrate, taurine (erythrocytes)	TMAO
Amphibian	Glycerol, glycerol (freezing)	Various α-amino acid,	GPC
Birds		Taurine(erythrocytes)	
Mammals			
- kidney	Sorbitol, *myo*-inositol	Taurine, others	Glycine betaine, GPC
- heart	*Myo*-inosito!	Taurine	
- brain		Taurine, glutamate	Glycine betaine, GPC

(Reproduced from Yancey, 1994)

2. Reproductive tract environment

Borland *et al.* (1977) reported the total concentration of ions in fluid obtained from the isthmus of the mice oviduct (35 hr post-coitus) which were detected by micropuncture and electron probe microanalysis. Osmolarity exceeded 360 mOsmol. The total concentration of Na^+, K^+, Mg^{2+}, Ca^{2+} and Cl^- were only 343 mOsmol and similar to values that obtained for fluid from human fallopian tube (Borland *et at.*, 1980). In contrast, Brinster (1965) studied the effect of osmolarity on the development of two-cell mouse embryos to blastocyst stage. The optimum osmolarity for development of two-cell embryos into blastocyst was 264 mOsmol whereas the embryo development significantly impaired at 354 mOsmol.

3. Amino acids in mammalian reproductive tract

The amino acid contents of rabbit eggs, preimplantation rabbit embryos and fluids of reproductive tract were investigated by the chromatography system. Glycine increased in reproductive tract fluids and embryos with development. Taurine was high in embryos and fluid but declined with development (except the blastocyst cellular compartment), while glycine levels rose. Leucine, valine, phenylalanine, and methionine were almost uniformly low in both embryos and reproductive tract fluids. Asparatic acid, serine, glutamic acid, and alanine were more prevalent and more likely to differ between development stages and between reproductive tract fluids and embryos (Miller and Schullz, 1987). The nutrient composition of mouse oviduct fluid was analyzed by an ultramicrofluorometric technique. The concentration of glutamine in the vicinity of the cumulus mass was 0.2 mM (Gardner and Leese, 1990). Changes in free six amino acid contents (alanine, aparatate, glutamate, glutamine, glycine, serine, and taurine) during development of blastocyst from two-cell mouse embryos *in vivo* were determined by HPLC analysis. The content of taurine and glutamine increased during development of Day 4 blasocyst from two-cell embryos. The content of glutamine decreased in blastocyst between Day 4 and 5, whereas the taurine content did not appear to change in

blastocysts when they approached implantation. The amounts of free glycine was high as taurine in two-cell embryos and decreased with development to Day 4 blastocyst stages, and this level remained low on Day 5 blastocyst stages (Van Winkle and Dickinson, 1995).

4. The role of amino acids as osmolyte in mammalian embryo in hypertonic fluid

Some amino acids found abundant in mouse and rabbit reproductive tract act as osmolyte in mammalian embryo. The effects of amino acids on the development on mouse embryos in high salt concentration medium were investigated. Raising the concentration of NaCl from 75 to 125 mM, in absence of glutamine, progressively inhibited development. In contrast, when included glutamine in the high NaCl concentration medium, glutamine protected against inhibition effect of high NaCl concentration (Lawitts and Biggers, 1992). The inhibitory effect of high concentrations of NaCl and the protective effect of betaine were studied by measuring the intracellular contents of potassium and sodium in pronuclear stage zygotes with X-ray electron probe spectrometry. When medium contain 85 mM or 125 mM NaCl, the intracellular content of sodium rose and the content of potassium decreased. However, these changes were partially reduced in the presence of 125 mM NaCl if betaine were also in the medium (Biggers, Lawitts and Lechene, 1993). Dumoulin *et al.* (1997) suggested that taurine was the osmolyte in mammalian embryos. They loaded [H^3] taurine into human oocytes (remained unfertilized after *in vitro* fertilization), human embryos (two- to eight-cell) and two-cell mouse embryos. These samples were incubated for 4 hr. in hypotonic medium (200 and 240 mOsmol/kg), the incubated samples showed significantly lower radioactivity as compare to samples which were incubated in medium of 280, 320, and 360 mOsmol/kg. The protective effects of several amino acids on the development of mouse zygotes into blastocyst stage were tested. Zygotes were cultured in 310 mOsmol medium with 130 mM NaCl, either without or with 1 mM of glutamine, taurine, hypotaurine, betaine, proline, glycine, sarcosine, GABA, myo-inositol, β-alanine,

lysine, and raffinose. Glutamine, betaine, proline, glycine and β-alanine were the compounds with the greatest and most consistent support of development to the ≥ four –cell stage and to the blastocyst as their responses were high ($p< 0.01$) compared to the control groups (Dawson and Baltz, 1997).

There are several experiments to propose that glycine can be the osmolyte in mouse embryo. A greater proportion of two-cell mouse embryos developed to blastocyst stage when 10 mM glycine was present in culture media in which its total concentration of other ions was 355 mM or more (Van Winkle *et al.*, 1990). To determine the amount of glycine accumulation, mouse zygotes were cultured in the medium contained [^{3}H] glycine at 250, 310 and 340 mOsmol. Glycine was accumulated at 310 and 340 mOsmol more than at 250 mOsmol ($p<0.001$)(Dawson, Collins, and Baltz, 1998). In eggs, zygotes and early cleavage-stage embryo of the mouse, it has been shown that uptake of glycine was mediated entirely by the Na^+- and Cl^- dependent Gly transporter (Hobbs and Kaye, 1985, 1986; and Van Winkle, 1988.) It was supported by finding that human cleavage-stage embryos also transport glycine via a Gly transporter (Hammer *et al.*, 2000).

In addition, Kolajova and Baltz (1999) demonstrated that mouse embryos also posses swelling - activated channels which function to release osmotically active glycine and other osmolytes when exposure to hypotonic medium. Glutamine, Glutamate, taurine, betaine, creatine, myo-inositol, glycerophosphorylcholine were found in rat tissues at 7 and 2 days prenatal (Miller, Hanson, and Yancey, 2000).

5. The effects of amino acids on the development of hamster embryos *in vitro*

There are several factors which affect the development of preimplantation hamster embryos. Amino acid is an important regulatory factor. Gwatkin and Haidri (1973) studied the requirements for the maturation of hamster oocytes *in vitro* and proposed that four amino acids, glutamine, isoleusine, phenylalanine, and methionine, were a nutritional need because they found that the omission of these four of Eagle's 13 amino acids (arginine, cysteine, glutamine, histidine, isoleusine, leucine, lysine,

methionine, phenylalanine, threonine, tryptophane, and valine) reduced development by more than 20%.

The beneficial effects of these four amino acids on the development of preimplantation hamster embryos were tested at the different hamster embryonic stages. Amino acids, cumulus cells, and bovine serum albumin were examined on *in vitro* fertilization and first cleavage of hamster eggs. In both cumulus-present and cumulus-free situations, addition of glutamine, isoleusine, phenylalanine, and methionine resulted a significantly higher proportion of eggs to cleave than when amino acids are not included (Juetten and Bavister, 1983). Moreover, in the unsupplemented culture medium, only 2% of early eight-cell embryos develop to blastocyst stage compared with 22% of late eight-cell embryos. In the presence of amino acids, 36% of early eight-cell embryos developed into blastocyst. The amino acids also increased the percentage of late eight-cell embryos. They develop into blastocyst from 22% to 66% (Bavister *et al.*, 1983).

However, two reports demonstrated that amino acids, alone and in combination, could stimulate or inhibit the hamster embryo development *in vitro* to blastocyst stage. Bavister and Arlotto (1990) studied the development of one-cell hamster embryos in culture medium (TLP-PVA) containing with single amino acids (cysteine-SH, arginine, phenylalnine, proline, valine, serine, threoine, histidine, alanine, isoleucine, hydroxyproline, glycine, leucine, cystine, lysine, aspartic acid, tryptophan, methionine, and tyrosine) compare to the control group. They proposed that phenylalaine, valine, isoleucine, tyrosine, trypophan, and arginine were the inhibitory amino acids, and the stimulatory amino acids were glycine, cystine, and lysine.

In addition, Mckiernan, Clayton, and Bavister (1995) also analyzed stimulatory and inhibitory effects of 20 amino acids (glutamic acid, cysteine, arginine, phenylalnine, proline, valine, serine, threoine, histidine, alanine, isoleucine, hydroxyproline, glycine, leucine, taurine, lysine, aspartic acid, tryptophan, methionine, and tyrosine) for the development of one-cell hamster embryos in HECM-3 with glutamine. The single amino acids with glutamine that stimulated the development of one-cell embryos to blastocyst

stage in vitro were taurine, glycine, serine, proline, histidine, asparagine, cysteine and lysine. The single amino acids with glutamine that inhibited the development of one-cell embryos to blastocyst stage *in vitro* were leucine, tyrosine, valine, isoleucine, phenylalanine, arginine, methionine, and cysteine. Combination of glycine, glutamine, and taurine plus the other eight amino acids; asparagine, aspartic acid, serine, glutamic acid, histidine, lysine, proline and cysteine significantly stimulated the development of one-cell embryos to blastocyst stage compare to combination of combination of glycine, taurine, and glutamine.

Glutamine is the one that influence strongly on the development of hamster embryo. From the study of Carney and Bavister (1985), only glutamine from four amino acids (Bavister *et al.*, 1983) was required for the development from the eight-cell to the blastocyst stage.

Kane, Carney, and Bavister (1986) compared the effect of glutamine, F10 amino acid, F10 vitamins, combination of glutamine and F10 vitamins, and combination of F10 amino acids and F10 vitamins on the development of eight-cell and morula hamster embryos. The control group was the unsupplemented medium. By 20 hr., morulae in medium supplemented with glutamine, glutamine + F10 vitamins, and F10 amino acids + F10 vitamins reached the blastocyst stage significantly (36%, 27% and 31%) when compared to the control group (18%). The eight-cell embryos in medium supplemented with glutamine, F10 amino acids, glutamine + F10 vitamins, and F10 amino acids + F10 vitamins developed to the blastocyst stage (21%, 25%, 27% and 26%.) significantly higher than the control group (8%). By 40 hr. in culture, both eight-cell embryos and morulae produced the proportion of hatched blastocyst in glutamine + F10 vitamins (8%, 10%) and F10 amino acids + F10 vitamins (10%, 9%) significantly higher in comparison to the control group (0%, 1%), glutamine (2%, 0%), F10 amino acids (1%, 0%), and F10 vitamins (0%, 1%).

Kane and Bavister (1988) suggested that glutamine was as efficient as all F10 amino acids in promoting blastocyst hatching of eight-cell embryo when included

polyvinylalcohol (PVA) in culture medium. Mckiernan, Tasca, and Bavister (1991) determined the requirements of pyruvate, lactate, and glutamine for development *in vitro* of one-cell and two-cell hamster embryos to blastocysts. Glutamine alone can not support development of one-cell embryos *in vitro* beyond the two-cell stage. In combination with pyruvate or lactate, glutamine increase the proportion of one-cell embryos developed to morulae/blastocysts. Glutamine alone support development of two-cell embryo to morulae/blastocysts at 32% while treatments contained either 0.2 mM or 1.0 mM glutamine with pyruvate and/or lactate enhance the development of embryo into morulae/blastocysts at 82-95%.

Taurine is another important amino acid. Taurine is analogous with hypotaurine. Taurine was required for the motility of hamster spermatozoa *in vitro* (Leibfried and Bavister, 1982). Since hypotaurine significantly improved development of one-cell embryos to eight-cell and further (range 22-27%) while none of embryos reached the eight-cell stage in the culture medium without hypotaurine (Barnett and Bavister, 1992). Thus taurine may support the development of hamster one-cell embryos into morulae and blastocysts *in vitro*.

6. The effect of osmolarity on the development of hamster embryos *in vitro*

Hamster embryos resist the stress from hypertonic medium in a limited range. Haidri and Gwatkin (1973) studied the maturation of hamster oocytes cultured in GH-1. No polar body formation occurred at 258 or at 263 mOsmol. At 268 mOsmol, 90 to 100% of the oocytes formed polar bodies. Polar-body formation declined above 268 mOsmol. At 278, 288 and 298 mOsmol, the percentage of oocytes maturing were 80 to 90, 70 to 80 and 20 to 30, respectively. Eight-cell embryos cultured in culture medium (TALP) developed to blastocyst stage between 225 to 300 mosmol/kg (Bavister *et al.*, 1983). Percentages of blastocyst development that developed from two-cell were highest at 250-300 mOsmol (51-54%). Percentages of blastocyst development at 350 mOsmol (8%) were significantly lower than at 250-300 mOsmol (Mckiernan and Bavister, 1990).