Positive effect of taurine on preimplantation development of mouse embryos *in vitro*

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Summary. The effect of various taurine concentrations in modified Tyrode's medium on *in vitro* fertilization of mouse oocytes was examined. No significant difference in fertilization rate was found at concentrations of 0, 0.1, 1, 5, 10 and 20 mM taurine. In a second series of experiments, the effect of taurine on preimplantation embryonic development after fertilization *in vitro* was studied. At concentrations of 1, 5, 10 and 20 mM taurine, significantly more two-cell embryos reached the blastocyst stage compared with medium without taurine. Culture in the presence of 5 mM or 10 mM taurine resulted in blastocysts with the highest mean number of cells. The positive effect of taurine on embryonic development was found to be more pronounced both in a second medium (human tubal fluid medium) which has a higher potassium concentration than Tyrode's medium, and in a modified Tyrode's medium with an increased potassium concentration. In addition to these *in vitro* studies, it is reported that taurine comprised about 59% of the total free amino acid content in mouse oviduct flushings, compared with 17% in mouse serum.

Keywords: taurine; *in vitro* fertilization; embryonic development; mouse

Introduction

Taurine and its precursor hypotaurine are sulfur-containing β -amino acids, and they are present at relatively high concentrations in female reproductive tract fluids of several mammals (Meizel *et al.*, 1980; Casslén, 1987; Miller & Schultz, 1987), in human semen (Hervann *et al.*, 1986) and in spermatozoal acrosomes (Velázquez *et al.*, 1986).

Much is already known about the role of taurine in the processes of sperm capacitation and *in vitro* fertilization (IVF) for different species. Hamster spermatozoa require taurine or hypotaurine in the culture medium to maintain their motility and viability during *in vitro* capacitation (Mrsny *et al.*, 1979). Also, the fertilizing capacity of hamster spermatozoa *in vitro* is enhanced by taurine and hypotaurine (Leibfried & Bavister, 1981, 1982). In the cow, the presence of hypotaurine in the medium stimulates *in vitro* fertilization (Ball *et al.*, 1983). However, mouse spermatozoa do not need the addition of taurine to exhibit their full fertilizing ability (Fraser, 1986). Although it has been reported that human spermatozoa capacitate faster in medium with taurine added (Mortimer, 1986; Chan, 1985), their fertilizing ability *in vitro* does not seem to be increased by taurine (Mahadevan & Trounson, 1985).

However, the role of taurine in mammalian embryonic development during the preimplantation stages is less clear. The purpose of the present study was first to examine the effect of taurine on *in vitro* culture of preimplantation mouse embryos. Second, reproductive tract fluids of female mice were analysed for the presence of taurine.

Materials and Methods

Modified Tyrode's medium (T6) (Quinn *et al.*, 1984) was used as culture medium. For *in vitro* fertilization, the medium was supplemented with 30 mg Fraction V bovine serum albumin/ml (BSA, Sigma No. A7906, St Louis, MO, USA); 5 mg BSA/ml was used for the embryo culture. Media with different taurine concentrations were prepared by mixing a stock solution of 20 mM taurine in culture medium (Sigma, No. T7146) with medium without taurine. The osmolality of all media was adjusted to 280–285 mOsm/kg before use, the pH was 7.5 after equilibration in an atmosphere of 5% CO₂ in air.

For all experiments (C57BL/6 × DBA/2)F₁ hybrid female mice (B6D2F1) were purchased from Charles River Wiga (Sulzfeld, Germany). Oocytes were obtained by superovulating 7–11-week-old females with 10 iu pregnant mares' serum gonadotrophin (PMSG, Sigma) and 48 h later 10 iu human chorionic gonadotrophin (hCG, Sigma). Sixteen hours after hCG injection, the females were killed by cervical dislocation. Their oviducts were dissected and transferred to Hepes-buffered T6 (Quinn *et al.*, 1984). The cumulus masses containing the oocytes were released from the swollen ampullae of the oviducts, washed once and transferred to Multidish 4-well dishes (Nunc, Life Technology BV, Breda, The Netherlands) containing 1 ml of the fertilization medium.

Caudal epididymal spermatozoa were obtained from 12–18-week-old CD-1 males. After incision of the tissues, the spermatozoa were allowed to disperse into T6 medium supplemented with 30 mg BSA/ml. After 30 min, the epididymal tissue was removed and the spermatozoa were allowed to capacitate for a further 90 min at 37°C. The oocytes were inseminated with a calculated amount of the sperm suspension to give a final concentration of approximately 2 000 000 motile sperm/ml.

Experiments to study the effect of taurine on fertilization and embryonic development *in vitro* were of a randomized block design (Fleiss, 1986). Oocytes from different females were not pooled, but were inseminated separately. The embryos from each female were distributed over a set of the different experimental media and were not pooled with embryos from other females. In this way, the comparison between different experimental media is repeated in every female used. To obtain at least five oocytes in every experimental medium from each female, only oocytes from females who produced > 30 oocytes were used.

Twenty-four hours after insemination, normal-appearing two-cell embryos, which were considered fertilized, were counted. At 120 h after insemination, the numbers of blastocysts were assessed. The percentages of embryos completing preimplantation development were calculated on the basis of the fertilization rate. The mean percentages ± standard error of the mean (SEM) of fertilization (number of two-cell embryos: number of oocytes) and of embryonic development (number of blastocysts: number of two-cell embryos) per female were calculated for every experimental group. Statistical analysis of the data was performed using an analysis of variance (ANOVA). When treatment effects were significant, multiple-comparison tests were used to determine which treatment means were different (Fleiss, 1986). In Expts 1 and 2, in which comparisons were made between groups cultured in the presence of several taurine concentrations and a control group cultured without taurine, Dunnett's procedure was used. In Expts 3 and 4, in which only a few comparisons between the group means were of interest, the Bonferroni criterion was used.

Experiment 1: dose–response of fertilization *in vitro* to taurine

Four replicate experiments using four to six females per experiment were performed. A sperm suspension from four males was divided among insemination media containing taurine in six different concentrations (0, 0.1, 1, 5, 10 and 20 mM) and capacitated for a further 90 min. Oocytes were freed from cumulus cells using hyaluronidase, and divided per female among the six insemination media. Five hours after insemination, oocytes of each experimental group were rinsed once and transferred to medium without taurine.

Experiment 2: dose–response of embryonic development to taurine

Five replicate experiments using five to nine females per experiment were performed. Media with the same taurine concentrations as in Expt 1 were used. Five hours after insemination in medium without taurine, oocytes were divided at random among medium without taurine and the five taurine-containing media. All the embryos, from two experiments, developing a distinct blastocoel at 120 h after insemination were fixed and stained with 4',6-diamidino-2-phenylindole (DAPI) according to the method of Mori *et al.* (1988). This DNA-staining technique allows an accurate count of the number of nuclei, and thus of the number of cells in each embryo.

Experiment 3: comparison of two culture media of different inorganic composition supplemented with taurine

In this experiment, T6 medium and human tubal fluid medium (HTF) (Quinn *et al.*, 1985) were used for the culture of embryos. Four replicate experiments using four to six females per experiment were performed. Five hours after insemination in T6 medium without taurine, the oocytes were randomly divided among T6 and HTF medium without taurine and T6 and HTF medium with 5 mM taurine.

Experiment 4: comparison of T6 medium with two K⁺ concentrations supplemented with taurine

Three replicate experiments using four to six females per experiment were performed using standard T6 medium (1.4 mM K⁺) and T6 with 5 mM K⁺ for the culture of embryos. Osmolality of the T6 with added K⁺ was adjusted to 280 mOsm/kg by replacing a small amount of medium by water. Five hours after insemination in T6 medium without taurine, the oocytes were randomly divided among T6 and T6 plus 5 mM K⁺, both with, and without, 5 mM taurine.

Experiment 5: the presence of taurine in mouse oviduct fluid

Mice were induced to ovulate by injection of 3 iu PMSG and then 3 iu HCG, 48 h later. Oviduct flushings and serum were collected 40 h after hCG injection. After anaesthetizing the mice with ether, blood samples were collected by puncture of the retro-orbital plexus. The blood was allowed to clot and serum was separated from cells by centrifugation (1200 g, 10 min). The mice were killed by cervical dislocation, and both oviducts together with a small portion of the uterus near the uterotubal junction (0.1–0.2 mm) were dissected. After rinsing the oviducts in 5% dextrose (Sigma) and blotting them on filter paper to remove most of the adhering peritoneal fluids, they were transferred to a drop of 150 µl dextrose solution. Part of this fluid was used to flush the oviducts using a plastic tuberculin syringe attached to a blunt hypodermic needle with an outer diameter of 0.21 mm (Hamilton N733, v. Oortmerssen, Den Haag, The Netherlands) introduced into the ampullar end of the oviduct. These manipulations were performed under a stereomicroscope. Flushings were completed within 10 min of death of the animal. The fluid used for flushing the oviducts was centrifuged at 2000 g for 10 min to remove any cellular debris and the supernatant was stored at –20°C.

Amino acid analysis was performed with an amino acid analyser (LKB/Biochrom model 4151, Cambridge, UK) according to the method of Blom & Huijman (1985) using norleucine as an internal standard.

Results

Experiment 1: dose–response of fertilization *in vitro* to taurine

From 20 females, 779 oocytes were obtained (mean number of oocytes $\pm$ standard deviation (SD) per female: 39.0 ± 5.6) and divided among the six media. No significant differences were observed between the number of two-cell embryos, counted at 24 h after insemination, in medium without taurine and the five media with different taurine concentrations (Table 1).

Table 1. Effect of taurine on the fertilization *in vitro* of mouse oocytes

Taurine (mM)	Two-cell formation (%) [*]	Mean fertilization rate per female $\pm$ SEM [†]
0	85:133 (64)	0.64 $\pm$ 0.05
0.1	79:126 (63)	0.62 $\pm$ 0.06
1.0	82:132 (62)	0.62 $\pm$ 0.05
5.0	83:130 (64)	0.64 $\pm$ 0.04
10.0	85:131 (65)	0.65 $\pm$ 0.06
20.0	74:127 (58)	0.59 $\pm$ 0.06

^{*}Total number of two-cell embryos:total number of oocytes.

[†]Means and SEM are based on 20 replicates: the oocytes of each of 20 females were divided among the six media with different taurine concentrations, inseminated and cultured to the two-cell stage separately. ANOVA: not significant.

Experiment 2: dose–response of embryonic development to taurine

For these experiments, 34 females were used. Five hours after insemination in medium without taurine, a mean number of 42.7 ± 10.5 oocytes per female (total number of oocytes: 1451) was divided among the six media. After 24 h, the numbers of two-cell embryos were not significantly

different in the six media (data not shown). Table 2 shows that, with respect to the mean number of blastocysts per female developing from two-cell embryos, the means of the groups cultured in media with taurine concentrations of 1–20 mM are significantly different from the mean of the control group (no taurine). The mean cell number of blastocysts, as estimated by the number of nuclei that developed in media with 5 mM and 10 mM taurine was significantly higher than in medium without taurine (Table 3).

Table 2. Effect of various taurine concentrations in the culture medium on the development of mouse embryos to the blastocyst stage

Taurine (mM)	Blastocyst formation (%) [*]	Mean blastocyst formation per female $\pm$ SEM [†]
0	99:165 (60)	0.64 $\pm$ 0.04
0.1	117:173 (68)	0.70 $\pm$ 0.04
1.0	131:167 (78)	0.82 $\pm$ 0.03§
5.0	121:157 (77)	0.79 $\pm$ 0.04§
10.0	117:153 (76)	0.80 $\pm$ 0.04§
20.0	130:165 (79)	0.80 $\pm$ 0.04§

^{*}Total number of blastocysts:total number of two-cell embryos.

[†]Means and SEM are based on 34 replicates: after *in vitro* fertilization in medium without taurine, the oocytes of each of 34 females were divided among the six media with different taurine concentrations and cultured separately.

§ANOVA: $P < 0.01$, treatment means indicated are significantly different from the control group.

Table 3. Cell numbers of mouse blastocysts after development of the embryos in T6 media with various taurine concentrations

Taurine (mM)	Number of blastocysts successfully analysed	Number of cells Mean $\pm$ SEM
0	33	54.7 $\pm$ 4.0
0.1	41	62.0 $\pm$ 3.5
1.0	36	52.6 $\pm$ 3.1
5.0	42	67.9 $\pm$ 3.4*
10.0	41	64.8 $\pm$ 3.2*
20.0	26	58.3 $\pm$ 4.1

*ANOVA: $P < 0.05$, treatment means indicated are significantly different from the control group.

Experiment 3: comparison of two culture media of different inorganic composition supplemented with taurine

For these experiments, 18 females were used. Five hours after insemination in medium without taurine, a mean number of 41.3 ± 8.4 (SD) oocytes per female (total number of oocytes: 744) was divided among the two types of culture medium, with and without 5 mM taurine. This concentration of taurine was chosen because it resulted in blastocysts with the highest mean number of cells (Table 3). After 24 h, the numbers of two-cell embryos were not significantly

different in the four groups (data not shown). For both types of medium, embryo development in taurine-supplemented medium resulted in a significantly higher blastocyst formation (Table 4).

Experiment 4: comparison of T6 medium with two K⁺ concentrations supplemented with taurine

For these experiments, 17 females were used. Five hours after insemination in medium without taurine, a mean number of 41.9 ± 6.4 (SD) oocytes per female (total number of oocytes: 712) was divided among the two T6 media with different K⁺ concentrations, with and without 5 mM taurine. The numbers of two-cell embryos 24 h after insemination were not significantly different in the four groups (data not shown). Embryonic development in T6 with 5 mM K⁺ without taurine was retarded (Table 5) as compared to T6 without extra potassium. For both K⁺ concentrations, embryo development in taurine-supplemented medium resulted in a significantly higher blastocyst formation (Table 5).

Table 4. Effect of 5 mM taurine in two media of different inorganic composition on the development of mouse zygotes to the blastocyst stage

Medium	Taurine (mM)	Blastocyst formation (%)*	Mean blastocyst formation per female $\pm$ SEM†
T6	0	80:119 (67)	0.64 ± 0.05
	5	93:116 (80)	$0.78 \pm 0.04§$
HTF	0	54:123 (44)	$0.43 \pm 0.05§$
	5	81:118 (69)	$0.70 \pm 0.04‡$

*Total number of blastocysts:total number of zygotes.

†Means and SEM are based on 18 replicates: the zygotes of each of 18 females were divided among the four media and cultured separately.

ANOVA: $P < 0.001$; §significantly different from T6 medium without taurine; ‡significantly different from HTF medium without taurine.

Table 5. Effect of 5 mM taurine in T6 media with different potassium levels on the development of mouse zygotes to the blastocyst stage

Potassium (mM)	Taurine (mM)	Blastocyst formation (%)*	Mean blastocyst formation per female $\pm$ SEM†
1.4	0	86:122 (70)	0.67 ± 0.08
1.4	5.0	108:129 (84)	$0.84 \pm 0.04§$
5.0	0	67:124 (54)	$0.51 \pm 0.06§$
5.0	5.0	91:122 (75)	$0.74 \pm 0.04‡$

*Total number of blastocysts:total number of zygotes.

†Means and SEM are based on 17 replicates: the zygotes of each of 17 females are divided among the four media and cultured separately.

ANOVA: $P < 0.001$; §significantly different from the mean blastocyst formation in T6 medium without taurine; ‡significantly different from T6 medium with extra K⁺ and without taurine.

Experiment 5: the presence of taurine in mouse oviduct fluid

Collection of nonhaemolysed serum and oviduct-flushing fluid was successful in five mice. In the 150 μ l fluid with which both oviducts of one mouse were flushed, a total mean concentration of 558 ± 121 μ mol/l free amino acids was found. The mean concentration of taurine in the oviduct-flushing samples was 329 ± 70 μ mol/l ($\pm$ SD). As oviduct fluid volume measurements were not

Table 6. Amino acid concentration and composition in serum and oviduct flushing fluid in mice

Amino acid	Concentration in serum Mean $\mu\text{mol/l} \pm \text{SD}$	Composition	
		Serum (% of total $\pm \text{SEM}$)*	Oviduct flushings (% of total $\pm \text{SEM}$)*
Taurine	590 $\pm$ 204	17	59 $\pm$ 1
Aspartic acid	18 $\pm$ 7	<1	4
Threonine	151 $\pm$ 47	4	1
Serine	169 $\pm$ 65	4	2
Glutamic acid	42 $\pm$ 16	1	9
Glutamine	766 $\pm$ 309	21 $\pm$ 1	5
Proline	79 $\pm$ 29		
Glycine	437 $\pm$ 173	12	10
Alanine	428 $\pm$ 136	12 $\pm$ 1	5
Valine	227 $\pm$ 79	6	1
Methionine	66 $\pm$ 22		
Isoleucine	112 $\pm$ 43	3	1
Leucine	163 $\pm$ 66	5	1
Tyrosine	63 $\pm$ 23		
Phenylalanine	74 $\pm$ 27	2	1
Ornithine	52 $\pm$ 21		
Lysine	389 $\pm$ 153	11 $\pm$ 1	1
Histidine	54 $\pm$ 19		
Arginine	176 $\pm$ 61		
Total	4055 $\pm$ 1451		

*The percentage of the total amino acid content measured (only amino acids that were found both in serum and in oviduct flushings are taken into account); SEM are not shown when they are below 0.5%.

made, only the relative composition (%) of amino acids found in the oviduct flushings is tabulated in Table 6, together with the relative composition of the same amino acids found in serum. Amino acids found in serum that could not be demonstrated in the oviduct flush samples were not taken into account. Although amino acid concentrations varied considerably among mice (e.g. minimum–maximum values of taurine concentration in serum and oviduct flushings of the five mice are 373–912 $\mu\text{mol/l}$ and 233–401 $\mu\text{mol/l}$, respectively), the variation in amino acid composition was very small, both in serum and in oviduct flushings.

Discussion

The results indicate that capacitation and IVF in the presence of taurine do not affect fertilization rate in mice at any of the concentrations tested. This is consistent with the observations of Fraser (1986), who reported that mouse spermatozoa do not require taurine to exhibit their maximum fertilizing ability and showed that, although addition of 0.1, 0.3 or 1.0 mM taurine in the absence of BSA resulted in higher fertilization rates, this increase was not found in media supplemented with BSA.

The inclusion of taurine in the culture medium resulted in significantly more two-cell embryos reaching the blastocyst stage for all tested taurine concentrations of 1 mM and higher. Embryos cultured in the presence of 5 and 10 mM taurine resulted in blastocysts with a higher mean number of cells compared with blastocysts cultured in medium without taurine.

To test whether the observed positive effect of taurine was a phenomenon specific for T6 culture medium, we compared the development of mouse embryos in T6 medium and HTF medium, with and without taurine. In the HTF medium, the positive effect of taurine was even more pronounced

than in T6 medium. HTF has been shown by Quinn *et al.* (1985) to be a more suitable medium than T6 for human IVF and for culturing mouse one-cell embryos. Contrary to the findings of Quinn *et al.*, comparing T6 and HTF without taurine, significantly fewer blastocysts developed in HTF medium than in T6 medium. Quinn *et al.* postulated that the high K^+ level (5.06 mM) in HTF medium compared with the K^+ level (1.42 mM) in T6 is responsible for the better results in the HTF medium. However, our results agree with the results of Wiley *et al.* (1986), who found that more blastocysts developed in T6 medium with 1.4 mM K^+ than in T6 with 6.0 mM K^+ .

Results from our investigation of the interactive effect between taurine and K^+ on the development of mouse embryos in T6 medium with the standard K^+ concentration and T6 medium with an increased (5.0 mM) K^+ concentration, both with and without taurine, also demonstrate that K^+ , and not some other component of the HTF medium, is responsible for the differences in embryonic development in T6 and HTF medium.

Taurine and K^+ are known to interact in sperm capacitation and fertilization processes (Meizel, 1985). Although their exact roles are not clear, both compounds seem to exert their effect by influencing the activity of the enzyme $Na^+-K^+-ATPase$. $Na^+-K^+-ATPase$ is a plasma-membrane enzyme that controls intracellular K^+ levels. The activity of this enzyme is inhibited in a low K^+ -environment (Thomas, 1972). Mrsny and Meizel (1985) showed that taurine decreases the $Na^+-K^+-ATPase$ activity of sperm cell membranes in a dose-dependent manner. High K^+ levels have been shown to be detrimental to sperm motility *in vitro* (Fraser, 1983). Studies by Meizel *et al.* (1980) have shown that, in the presence of 3.1 mM K^+ , addition of 0.5 mM taurine is needed to preserve hamster sperm motility during *in vitro* capacitation. In medium with a very low K^+ concentration, the addition of taurine is not needed (Mrsny & Meizel, 1985). It has been suggested that taurine may protect the spermatozoa against high K^+ concentrations by reducing the $Na^+-K^+-ATPase$ activity and consequently the influx of extracellular K^+ (Mrsny & Meizel, 1985).

Both taurine and K^+ are present in high concentrations in mammalian reproductive tract fluids. We found in the mouse that taurine comprised about 17% of the total free amino acid content in serum, whereas it amounted to 59% in the oviduct flushing samples. The relative proportions of the amino acids found in serum are comparable to those found by Schultz *et al.* (1981). Human uterine fluid has also been shown to contain high concentrations of taurine, varying from 3 to 17 mmol/l, i.e. up to 400-fold higher than that in blood plasma (Casslén, 1987). The concentration of taurine is highest during the luteal phase of the menstrual cycle (Casslén, 1987). The K^+ concentration in human uterine fluid has been shown to be approximately 20 mM at midcycle, which is about five times higher than that in human plasma (Casslén, 1984). Different K^+ concentrations in the human oviduct have been reported. Lippes *et al.* (1972) found K^+ concentrations between 7.7 mM and 9.9 mM, depending on the stage of the menstrual cycle, whereas Borland *et al.* (1980) found a mean concentration of 21 mM K^+ . In the mouse, the K^+ concentration in oviduct fluid was found to vary from 18 mM to 30 mM, depending on the stage of the oestrous cycle and the segment of the oviduct studied (Borland *et al.*, 1977; Roblero *et al.*, 1976). During pseudopregnancy, mouse uterine fluid has a very high K^+ content, ranging from 35 mM on day 1 to 75 mM on day 5 (Wales & Edirisinghe, 1989).

The high combined concentration of taurine and K^+ in reproductive tract fluids suggests that, *in vivo*, taurine might also protect the spermatozoa from the high K^+ levels by reducing the $Na^+-K^+-ATPase$ activity (Mrsny & Meizel, 1985). Not only do mammalian sperm capacitation and fertilization take place in uterine and oviductal fluids, but these fluids also make up the environment in which the embryo develops during the preimplantation stages. It is possible that taurine influences embryonic development in the same manner as it influences sperm motility, capacitation and fertilization, i.e. by protection against the high K^+ concentrations found in reproductive tract fluids. Findings of a greater stimulating effect of taurine in the media containing higher K^+ concentration are consistent with such a hypothesis. It remains to be shown whether the favourable role of taurine in embryonic development is exerted by protecting embryos against high K^+ concentrations by decreasing the $Na^+-K^+-ATPase$ activity of embryonic membranes.

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