—Mini Review—

The early history of the TYH medium for *in vitro* fertilization of mouse ova

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Abstract: The TYH medium was first reported as a medium for *in vitro* fertilization (IVF) of mouse eggs with epididymal spermatozoa, by Toyoda, Yokoyama and Hosi, in 1971. It was a modified Krebs-Ringer bicarbonate solution, supplemented with glucose, Na-pyruvate, antibiotics and bovine serum albumin. In TYH medium, almost all eggs are fertilized within 1 h, if the spermatozoa are pre-incubated for 2 h before insemination, while the sperm penetration is delayed for approximately 1 h when fresh epididymal spermatozoa are used. These findings showed that sperm capacitation can be induced in a chemically defined medium without requiring the female reproductive tract. Although the medium was not specifically named in the original paper, it was later called "TYH" after the initials of the three authors of the original paper. The IVF method using TYH medium is widely used due to its high reproducibility. In this mini-review, we describe the early efforts to develop the TYH medium and briefly discuss the related areas.

Key words: *In vitro* fertilization (IVF), Capacitation, Sperm, Egg, Mouse

Introduction

Nearly half a century has passed since the initial publications on mouse *in vitro* fertilization (IVF) by Toyoda, Yokoyama and Hosi [1, 2], in the *Japanese Journal of Animal Reproduction*(JJAR, which was succeeded by the J*ournal of Reproduction and Development* in 1977 as the official journal of the Society for Reproduction and Development), in June 1971. These studies were carried out in the School of Veterinary Medicine and Animal Science, Kitasato University, Towada, Japan, partly supported by a Grant (No.86586) from the Ministry of Education of Japan, to the late Professor Tosiro Hosi (1907–1989). The authors are deeply grateful to him for his encouragement during the course of these studies.

Historical Background

The first cytological evidence of IVF was obtained in 1954 by Charles Thibault and his colleagues in France, using recently ovulated eggs and 'uterine spermatozoa' recovered from the uterus of mated rabbits. This experiment was based on the independent discovery of capacitation by Austin and Chang in 1951. Chang [3] then obtained live offspring by transferring IVF rabbit embryos that had developed to the 4-cell stage in culture, thus providing definitive proof of the normal developmental capability of IVF embryos (for further details, see the reviews by Austin [4], Chang [5] and Thibault [6]).

In 1963 and 1964, Yanagimachi and Chang [7, 8] showed that golden hamster eggs can be fertilized by epididymal spermatozoa, in a micro-drop culture composed of cumulus-enclosed ovulated eggs and a small amount of sperm suspension in Tyrode's solution under mineral oil, thus suggesting the potential for *in vitro* capacitation in mammalian species for the first time. This landmark finding revolutionized the methodology of IVF, resulting in a burst of research during the 1960s through the 1970s.

The first successful mouse IVF was achieved by Whittingham in 1968 [9], using uterine spermatozoa collected 1 to 2 h after mating. He obtained live fetuses on day 17 of gestation, following the transfer of IVF embryos at the 2-cell stage to pseudo-pregnant recipient mice. It was the first report of successful IVF/embryo transfer (ET) in the mouse, although the birth of normal mice from IVF embryos was not described in his paper, and was first reported by Mukherjee and Cohen in 1970 [10]. The me-

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Table 1. Composition of Media TYH, TYH-280, and c-TYH

Component	Concentration in Medium (mM)		
	TYH	TYH-280	c-TYH
NaCl	119.37	72.13	119.37
KCl	4.78	4.78	4.78
$CaCl_2 \cdot 2H_2O$	1.71	–	1.71
Ca lactate	–	1.71	–
KH_2PO_4	1.19	1.19	1.19
$MgSO_4 \cdot 7H_2O$	1.19	0.85	1.19
$NaHCO_3$	25.07	25.07	25.07
Na lactate	-	30	–
Na pyruvate	1	0.3	0.5
Glucose	5.56	5.56	5.56
Bovine Serum Albumin	4 mg/ml	4 mg/ml	–
Methyl-beta-cyclodextrin	–	–	0.75
Polyvinyl-alcohol	–	–	1 mg/ml
Penicillin G potassium	7.5 mg/100 ml	7.5 mg/100 ml	7.5 mg/100 ml
Streptomycin sulfate	5.0 mg/100 ml	5.0 mg/100 ml	5.0 mg/100 ml
Phenol red	0.2 mg/100 ml	0.2mg/100 ml	0.2mg/100 ml
Reference	1	50	39

dium used for IVF by Whittingham was a modified Krebs-Ringer bicarbonate solution supplemented with glucose, Na-pyruvate, Na-lactate, bovine serum albumin and antibiotics, developed by Whitten and Biggers [11] for mouse embryo culture from the 1-cell zygote to the blastocyst stage. In 1969, Iwamatsu and Chang [12] reported the first successful mouse IVF by epididymal spermatozoa. Successful IVF of Chinese hamster eggs with epididymal spermatozoa was also reported by Pickworth and Chang in 1969 [13].

In the same year, Edwards *et al*. [14] reported successful IVF of human preovulatory oocytes using washed, ejaculated spermatozoa, after adapting the conditions for hamster IVF developed by Bavister [15]. In a monumental lecture at the 7th World Congress on Fertility and Sterility, held in Kyoto, October 1971, Edwards [16] stated that "For a long time it was believed that uterine spermatozoa had to be used to achieve fertilization *in vitro*, and even the observation by Yanagimachi and Chang (1964) that epididymal spermatozoa could be used for the fertilization of hamster eggs *in vitro* seemed hardly to shake the belief in the necessity of uterine spermatozoa in other species".

It was during these controversial periods that we began our search for a repeatable procedure for mouse IVF. As Iwamatsu and Chang had already succeeded in mouse IVF by epididymal spermatozoa in the presence of bovine follicular fluid [12], our purpose was to develop an IVF procedure without the need of fluids from the female reproductive tract.

TYH in the Cradle

The preliminary results of the experiments were presented at the 11th Annual Meeting of the Japanese Society of Mammalian Ova Research on April 6, 1970, by Toyoda and Yokoyama, entitled simply "Fertilization of mouse eggs *in vitro*" in Japanese [17]. In their presentation, superovulated eggs from mature females were co-incubated with epididymal or uterine spermatozoa under 5%CO_2 in air at 37 °C in a modified Krebs-Ringer bicarbonate medium (Table 1) according to Biggers *et al*. [18]. ICR-JCL mice were used throughout these early studies. The fertilization rates with epididymal sperm were quite variable, ranging from 34% to 100%, with a mean of 44.0%, which was significantly lower than that of uterine spermatozoa, 86.0% (85%-100%). It was noticed, however, that highly synchronous sperm penetration into eggs was occasionally observed, especially when the timing of insemination was unintentionally delayed, so that the sperm were left in the medium for a longer period before being used for insemination. After refining the methods for nearly one year, the manuscripts of two papers on TYH were submitted to the Editor of JJAR on January 18, 1971.

Overseas, progress was rapid during these periods and two important papers appeared in November and December, 1970. One was a further study of mouse sperm capacitation by Iwamatsu and Chang [19] who showed that the sperm penetration into the egg occurs

earlier when the sperm are pre-incubated in bovine follicular fluid. An attempt to incubate the sperm without bovine follicular fluid was not presented, however.

The other study was by Cross and Brinster (1970) [20] on the *in vitro* development of mouse oocytes. The composition of their medium for *in vitro* maturation of mouse oocytes (modified BMOC-2) was almost the same as that of TYH, although they followed the Whittingham's procedure [9] for IVF of *in vitro* matured oocytes using uterine spermatozoa. The largest difference between the modified BMOC-2 and TYH was the concentration of bovine serum albumin (BSA): 30 mg/ml in the modified BMOC-2 and 4 mg/ml in the TYH. Compared to the composition of embryo culture medium [11], both modified BMOC-2 and TYH do not contain lactate and have increased amounts of NaCl and pyruvate.

Capacitation *in vitro*

In our experiments, almost all eggs were fertilized within 1 h, if the spermatozoa were previously incubated in another droplet of TYH for 2 h before insemination, while sperm penetration was delayed for approximately 1 h when fresh sperm were used. The delay was only 1 h, but the difference was highly significant. This indicated that achieving IVF without any obvious sperm pretreatment does not necessarily mean that capacitation is not needed. Capacitation may proceed during co-incubation with eggs resulting in successful fertilization, if the eggs retain their ability to be fertilized. This notion was supported by Inoue and Wolf [21] who showed that fresh sperm binding to the zona pellucida of unfertilized, cumulus-free mouse eggs is a time-dependent process reflecting the occurrence of capacitation during culture.

Fukuda *et al*. [22] reported the successful fertilization of cumulus-free (denuded) mouse eggs by pre-incubated spermatozoa as the third paper of mouse IVF in TYH. Their results showed that there was no significant difference either in the fertilization rate or in the incidence of polyspermy between intact and denuded eggs. The role of cumulus cells in fertilization was re-evaluated later by Itagaki and Toyoda [23] and by Takahashi *et al.* [24].

Although the mouse strain used in the early experiments was exclusively a closed colony of ICR-JCL, the same technology was successfully applied to several inbred strains, including BALB/c, C3H/He, C57BL/6 J, DDD, and NC, resulting in high fertilization rates (more than 80%) in all the strains examined, except for a slightly lower rate (68.0%) when C3H/He eggs were fertilized with BALB/c spermatozoa (Yokoyama and Tanioka, 1977) [25].

Another IVF medium for mouse sperm capacitation and fertilization was developed by Lynn R. Fraser and Linda M. Drury in 1975 [26]. Their medium is a modified Tyrode's solution supplemented with glucose, Na-pyruvate, Na-lactate, antibiotics and serum albumin, usually bovine, following the formulation of Whittingham for culturing mouse ova [27]. The essential role of glucose for sperm penetration into mouse eggs in vitro was reported by Fraser and Quinn [28] and by Okamoto and Toyoda [29] in their respective media. One of the differences between the two methods was that the sperm concentrations during pre-incubation and fertilization were about 10 times higher in Fraser's medium (~$1–4 \times 10^7$ /ml and $1–4 \times 10^6$ /ml) than those of TYH (~$1–2 \times 10^6$ /ml and $1–5 \times 10^5$ /ml) [30].

In our system, penetration through the zona pellucida by more than one sperm is frequently observed, especially when 2h-preincubated spermatozoa are used. However, the rate of polyspermic fertilization, as judged by the number of male pronuclei and/or enlarged sperm heads in the vitellus, remains relatively low (8.2%, 39/477) [2]. In a series of cytogenetic studies on IVF mouse embryos, Yoshizawa *et al.* [31] employed TYH as a control medium and reported that 82.2% (244/297) of 1-cell embryos with mitotic figures have normal 2n diploid chromosomes at the first cleavage. They used superovulated eggs from F1 (BALB/c x C57BL/6) female mice and epididymal spermatozoa from ICR male mice, following pre-incubation for 2 h. Cytogenetics of IVF embryos produced in TYH was further analyzed by Tateno and Kamiguchi [32] in their studies on the chromosomal risk following intracytoplasmic sperm injection (ICSI) in the mouse. They used B6D2F1 hybrid mice to collect oocytes and spermatozoa and found that the incidence of IVF embryos with structural chromosome aberration was only 2% (8/355) at the first cleavage. An interesting finding in ICSI embryos was that the aberration rate was not significantly different from that of control embryos, when the spermatozoa were previously incubated for 2 h or more in TYH medium before being used for injection, while the incidence was increased in a time-dependent manner in other culture conditions. In this experiment, the IVF and ICSI eggs were transferred to modified CZB medium [33] at 2 h after insemination.

TYH as a Chemically Defined Medium

The significance of the TYH medium was first evaluated internationally by Dr. R. G. Edwards as the Editor of the *Research in Reproduction*, in November 1971 [34]. He introduced our papers that "The capacitation of

mouse spermatozoa *in vitro* has been recently achieved by Toyoda, Yokoyama and Hosi", and concluded: "This evidence indicates that mouse spermatozoa can be capacitated *in vitro* in a chemically defined medium without the necessity of fluids from the female reproductive tracts".

This definition was criticized later, however, because the medium was not completely chemically defined, at least from two view-points. The first criticism was raised by Gwatkin in 1977. In his monograph, entitled "Fertilization Mechanisms in Man and Mammals" [35], he commented on the TYH medium, writing that "Since contribution of epididymal secretions was ignored, capacitation could not be said to have occurred in a chemically defined medium as authors claimed". It was difficult to dispute this comment directly, because epididymal sperm were allowed to disperse in TYH medium for pre-incubation and were not "washed" as in the case of denuded eggs, but an experiment with a special congenic strain, B10. BR-Y^{del}, provided an answer [36]. This strain is characterized by a partially deleted Y chromosome, with higher rate of abnormal spermatozoa than its control strain. The low IVF rate of this strain in TYH medium is markedly improved when the sperm suspension is "washed" by Percoll gradient centrifugation. Improvement in the fertilization rate after removal of epididymal secretions was also observed by Fraser and Drury (1976), who used BALB/c eggs and spermatozoa for IVF in their medium [37]. It was inferred that the improvement was due to the removal of "decapacitation factor(s)" in the epididymal fluid.

The second criticism was based on the presence of serum albumin in the medium, which is an essential component for achieving IVF. As the quality of BSA varied considerably among products, there remained the worry that the beneficial effects might have been due to some unknown contaminants in the serum. At the 13th Annual Meeting of the Society of Mammalian Ova Research in 1972, Yokoyama and Toyoda [38] reported that fertilization rates of more than 80% were achieved with various preparations of serum albumin, not only of bovine but also of horse, rabbit, chicken, and human, while other macromolecules, such as chicken ovalbumin, dextran, or polyvinylpyrrolidone, are totally ineffective. Recently, the quality of BSA has become much improved, so that we are confident that its essential role is due to the property of the molecule itself, not unknown contaminants.

In 1998, Choi and Toyoda [39] made a protein-free version of TYH (c-TYH) medium, in which BSA was substituted by methyl-beta-cyclodextrin and polyvinyl-alcohol (Table 1). Cumulus-free mouse eggs, fertilized by spermatozoa treated with 0.75 mM methyl-beta-cyclodextrin, developed to the blastocyst stage when cultured in kSOM [40] (45%, 36/80), and to live offspring following transferred to recipients at the 2-cell stage (38%, 62/160). This medium was originally devised for analyzing the role of cholesterol removal from sperm cells during capacitation, but it was shown to be especially effective for the capacitation of frozen-thawed mouse spermatozoa by Takeo *et al*. [41].

Development of IVF Embryos

In ICR mouse eggs fertilized by pre-incubated sperm in TYH medium, the second meiotic division resumes within 1 h of insemination, and reaches telophase II together with an enlarged sperm head in the vitellus. Most eggs are at the pronuclear stage 3–4 h after insemination. When these eggs are left in the medium overnight, some of them develop to the 2-cell stage, but about half of them degenerate. An interesting observation was made by Osamu Okada, a graduate student in our laboratory at that time. He noticed that the development to the 2-cell stage increased significantly by increasing the volume of fertilization medium. It was usual at that time, and even still now, to introduce the eggs from both oviducts of a female mouse into a 0.4 ml droplet of TYH medium, but Okada increased the volume to 0.8ml per droplet for each oviduct, that is four times larger than the control. The developmental rate to the 2-cell stage jumped from 55.0% to 85.1%. This observation was presented at the 14th Annual Meeting of the Japanese Society of Mammalian Ova Research on April 6, 1973 by Okada [42]. The degeneration was considered to have been caused by some deleterious substance (s) liberated from dispersed cumulus cells and/or dead spermatozoa left in the medium, or due to depletion of some essential component (s) of the medium, most probably pyruvate.

Since that time, it has become routine to wash pronuclear eggs and transfer them to a new droplet of medium at 5–6 h after insemination for further development [43]. In this condition, the first cleavage regularly occurs at 17–18 h after insemination in the presence of adequate amounts of pyruvate, in good agreement with the time reported by Kaufman [44]. The first cleavage was retarded when the level of pyruvate in the TYH medium was reduced to 0.125 mM and all of the eggs degenerated in the absence of pyruvate, as reported by Biggers *et al*. [18] for 1-cell mouse zygotes fertilized *in vivo*.

Development beyond the 2-cell stage was another challenge. It was rare for the IVF embryos of the ICR strain to proceed to the 4-cell stage in TYH. One way to alleviate this block was to transfer the embryos back to

the maternal environment. Using this method, Okada obtained live offspring derived from 2-cell embryos fertilized in 0.8 ml culture [45]. In that experiment, a total of 144 two-cell embryos were transferred to 14 pseudo-pregnant recipients, resulting in the birth of 10 live offspring from 4 recipients 19–20 days after transfer. Although the rate of development (10/144) was significantly lower than that of control *(in vivo*-fertilized) 2-cell embryos (26/51), all the young, except for one lost during the nursing period, grew up normally.

Before Okada's experiment, Miyamoto and Chang [46] had obtained IVF embryos derived from superovulated eggs and epididymal spermatozoa from mature Swiss albino mice, by slightly modifying TYH medium. The rate of development into normal fetuses was 13% (10/80), on the 17th day after the transfer of 2-cell embryos. They wrote in their paper: "There is no doubt that the normal development of mouse eggs fertilized *in vitro* by epididymal spermatozoa is possible, although the potential for development still awaits further improvement."

More recently, Yokoyama *et al.* [47] established IVF/ET technology as a means of supporting the production of offspring in mouse strains with reproductive disorders, especially in mouse models of various human diseases, such as progressive muscular dystrophy (C57BL/6-*dy/dy*), obesity (C57BL/6-*ob/ob*), and motor ataxia (BALB/c-*rl/rl*, BALB/c-*shi/shi*, and C57BL/6-*mld/mld*). Moreover, IVF/ET has become a key technology in keeping genetic resources in "mouse embryo banks," combined with cryopreservation technologies of gametes and embryos [48, 49].

Notes on the Preimplantation Embryo Culture

As there have been so many reports on the culture media for preimplantation embryonic development, only brief notes directly related to the TYH medium are presented here.

In 1978, Kasai *et al.* [50] published description of a modified version of the TYH medium (later called "TYH-280", Table 1), which has a composition much closer to that of the embryo culture medium of Whitten and Biggers [11]. They reported that all the processes from sperm capacitation through to fertilization, and the development up to the blastocyst stage could be performed in the new medium. Superovulated eggs from three inbred strains (BALB/c, C57BL/6 J-at/at, and C3H/He) and their F1 hybrids were inseminated with ICR-JCL epididymal spermatozoa pre-incubated for 1 h, and the eggs undergoing fertilization were transferred to a new droplet under oil at 6 h after insemination, then cultured for an additional 90 h at 37 °C, under 5% CO_2 in air. Although about 30–54% of the 2-cell embryos from F1 females developed to blastocysts, the development of embryos from the three inbred strains was extremely low (0–3% development to blastocyst).

This obstacle was partly overcome by Hoshi and Toyoda [51] by introducing a small amount (0.1 mM) of EDTA to the embryo culture medium, following the observation of Abramczuk *et al.* [52] that this chelating compound has beneficial effects on the preimplantation development of mouse zygotes fertilized *in vivo.* Following the publication of these results, the two-step procedure became standardized, first by using TYH medium for capacitation and fertilization, and then using THY-280 or Whitten's medium supplemented with EDTA (mWM) for preimplantation development. The feasibility of this procedure was confirmed by Suzuki and Toyoda (1986) [53], who showed that normal young can be obtained by transferring IVF embryos developed in mWM to the blastocyst stage.

Another successful result was reported by Nakagata and Tanaka [54] who used pre-ovulatory mouse oocytes, as a model of human IVF. They obtained pre-ovulatory oocytes from the ovaries of C57BL/6 mature mice at 10 h after human chorionic gonadotropin (HCG) injection, incubated them for 2–6 h, and then inseminated them with pre-incubated ICR-JCL spermatozoa in TYH medium. The eggs were transferred to TYH-280 medium supplemented with 0.1 mM EDTA at 5 h after insemination and further incubated for 96 h. It was found that the incubation of pre-ovulatory oocytes before insemination significantly improved their development to the blastocyst stage, from 40.8% without incubation to 90.1% in 6h-incubated oocytes, a proportion which was not significantly different from that of control (ovulated) oocytes collected from the oviducts 16 h after administering HCG (96.7%).

The reliability of the TYH/mWM system was further supported by Naito *et al.* [55] for the production of normal mice from zona pellucida-free mouse eggs fertilized and developed up to the blastocyst stage *in vitro* and then transferred to the recipients.

Concluding Remarks

In addition to the early reports of successful IVF described above, IVF in various laboratory animal species was achieved during the 1970s, first by using uterine spermatozoa, in the cat [56] and rat [57], and then with epididymal spermatozoa, in the guinea pig [58], rabbit [59], rat [60, 61] and two species of deer mouse [62]. The importance of sperm pre-incubation for the capacitation

was also shown by Toyoda and Chang [63] in the rat, and by Hosoi *et al*. [64] using rabbit epididymal spermatozoa. Furthermore, IVF with ejaculated spermatozoa has been achieved in the squirrel monkey [65], rabbit [66], rat and mouse [67], and dog [68], and by spermatozoa from the ductus deferens in the cat [69].

The various methods so far devised for IVF may reflect diverse phylogenetic strategies for maximizing the chance of fertilization in different mammalian species, or simply reflect an inadequate understanding of the process of fertilization *in vivo*. One of the sources of confusion in early studies, pointed out by Edwards [16], is that the uterus was regarded as the major site of capacitation. The journey of spermatozoa toward the site of fertilization (ampulla of the oviduct) is far more complicated than previously thought, as reviewed by Suarez and Pacey [70]. It is expected that the roles of the microenvironment of the female reproductive tract will need to be clarified further in efforts to improve assisted reproductive technologies [71].

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