ANIMAL EXPERIMENTATION

Stimulation of Early Embryonic Development in Cattle by Coculture with Surfactant

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Purpose: Our purpose was to determine the efficacy of Surfacten, a bovine pulmonary surfactant, on the maturation of in vitro bovine ova.

Methods: We used Surfacten as a supplement to the coculture media both at the onset of coculture and after cleavage in bovine ova had been determined. The controls received no Surfacten.

Results: The maturation rate in bovine embryos to the blastocyst stage statistically improved ($P < 0.05$) in the series in which Surfacten was added to the media at the onset of coculture, compared with the controls and the series in which Surfacten was added after cleavage had been determined.

Conclusions: Surfacten, a commercially available surfactant which is a naturally occurring phospholipid that dramatically increases in the cervical mucus and the ampullaris of the oviduct at or near the time of ovulation, improves the maturation of bovine embryos in vitro by making the coculture medium approach the conditions found in the oviducts.

KEY WORDS: bovine embryos; coculture media; pulmonary surfactant.

INTRODUCTION

We identified a phospholipid in the internal genital tract that closely resembles pulmonary surfactant. This material closely resembles pulmonary surfactant morphologically, histochemically, and physiologically, and

thus we have come to label it the surfactant-like material in the female genital tract (1). This surfactant-like material reacts physically in response to the menstrual cycle and is most abundant at or about the time of ovulation. Because of this increased abundance at the period of highest fertility, we assumed that it must play some role in fertility expression. This assumption is further supported by the fact that it is found at a high concentration in the ampullar portions of the fallopian tubes and the uterine cervical mucus. To determine the role of this material we added it to the culture medium before the introduction of sperm and after fertilization had been established.

MATERIALS AND METHODS

Surfacten (Tokyo Tanabe Pharmaceuticals, Tokyo) is a surfactant obtained from bovine lung tissue. Bovine granulosa theca cells and ova were obtained at the time of ovum retrieval for in vitro fertilization. Frozen bovine sperm was thawed and motility confirmed before it was added to the culture media. We divided our trial series into four groups: (A) controls were conducted in six trials on 255 fertilized ova determined by evidence of cleavage, with the addition of granulosa theca cells but no surfactant, (B) surfacten was added to the culture medium after fertilization was achieved. This was performed in a series of six trials on 247 of the cleaved ova, and (C) surfacten (10 $\mu\text{g/ml}$) was included throughout the entire incubation in a series of five trials on 201 cleaved ova. (D) Surfacten (100 $\mu\text{g/ml}$) was included throughout incubation of 256 fertilized ova in a series of six trials.

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The procedures involved retrieval of viable ova from healthy mature cows which were precultured for 24 hr. It was at this time that Surfacten was added to the culture medium in groups C (10 $\mu\text{g/ml}$) and D (100 $\mu\text{g/ml}$), prior to the introduction of the thawed bovine sperm. This combination of the Surfacten containing the medium and the eggs and sperm was cocultured for 48 hr, and the number of zygotes at the two- and four-cell stage evaluated. Group B had the Surfacten introduced into the medium after 48 hr of coculture, and group A received no Surfacten. All groups were further incubated and the progression to blastocysts was evaluated.

RESULTS

Routine coculturing with granulosa thecal cells of extracted bovine ova without Surfacten in six trials with 255 fertilized ova displayed development to blastocysts in 23.9%. Coculturing with the addition of Surfacten on observing cleavage in six trials involving 247 bovine ova resulted in blastocyst formation in 51, giving a rate of 20.6%. In five trials in which Surfacten (10 $\mu\text{g/ml}$) was present throughout the entire coculturing, of 201 ova which displayed cleavage, 55 went on to form blastocysts, giving a rate of 27.3%. In six trials where Surfacten (100 $\mu\text{g/ml}$) was present throughout the entire coculturing, of 256 ova which displayed cleavage, 83 went on to form blastocysts, giving a rate of 32.4%. Thus, even at a lower concentration, Surfacten, if included early enough to allow it to aid in the capacitation of spermatozoa, results in improved progression from cleavage to the blastocyst stage ($P < 0.05$) (Table I).

Table I. The Effect of Surfacten on the Development of Bovine Embryos Undergoing IVF^a

	No. of expts	No. of cleaved ova cultured	No. of blastocysts (blastocysts/cleaved ova)
(a) Controls	6	255	61 (23.9%)
(b) Surfacten	6	247	51 (20.6%)
(c) Surfacten	5	201	55 (27.3%)
(d) Surfacten	6	256	83 (32.4%)

^a (a) Controls with no Surfacten in the culture medium. (b) Surfacten was added after fertilization, though a and b showed no significant difference. (c, d) Surfacten was added throughout the time of culture. Final Surfacten concentration—(b, c) 10 $\mu\text{g/ml}$ and (d) 100 $\mu\text{g/ml}$; both showed a significant improvement over a and b ($P < 0.05$).

DISCUSSION

The surfactant-like material found in the female genital tract consists of various phospholipids and is difficult to distinguish from pulmonary surfactant. Morphologically the material, which is excreted by a merocrine process, is in the form of granules which closely resemble the images of surfactant on electron micrographs (Figs. 1 and 2). The phospholipid constituents are in close agreement with those found in pulmonary surfactant (Table II). Our investigation revealed that Surfacten, when added to the culture medium prior to the introduction of sperm, resulted in enhanced development of zygotes to the blastocyst stage, compared to cultures which received no Surfacten or those in which Surfacten was added to the culture medium after fertilization had been achieved (two- to four-cell stage).

Our results indicate that when Surfacten is added throughout the coculture period, the ova which undergo cleavage then progress to the blastocyst stage at a better percentage than when Surfacten is not added to

Fig. 1. A transmission electron micrograph showing the surfactant-like material being extruded from a cell by a merocrine process. More granules of the material can be seen close to the cellular wall. This photo was taken during the ovulatory phase at the level of the tubal isthmus (human tubal lining). Original magnification, $\times 12400$.

Fig. 2. The lamellar structure of the surfactant-like material. A transmission electron micrograph of granules of the surfactant-like material in the ampullar portion of the tube during the ovulatory phase. The lamellar structure of the granule was delineated by high electron density at a wavelength of 45–50 Å. Original magnification, $\times 35000$.

the medium (controls) or when it is added after cleavage has been determined (2). Many authors have reported on the improved progression to the blastocyst (hatching) stage in the presence of oviductal tissues in the coculture medium. For instance Bongso *et al.* report on the improved quality of human embryos when cultured with human ampullary cells (3). Carney *et al.* compared coculturing with oviduct epithelial cells and other somatic cells in rabbits (4). Yeung *et al.* report that in vitro, human embryos achieve improved development when cocultured with human oviductal cells (5). Bongso *et al.* report an improved pregnancy rate with embryos grown in human fallopian tubal cell coculture (6). Many other authors have reported on cattle, sheep, and mice (7–9). Our approach was to investigate one purified material from the female genital tract, the so called surfactant-like material. This phospholipid, which closely resembles pulmonary surfactant, is not commercially available, necessitating the use of an available surfactant derived from bovine pulmonary tissues. Surfacten, added at the onset of coculture is beneficial in inducing bovine ova at the

Table II. Composition of Human Tubal Secretions (Surfactant-like Material)

	Follicular phase ^a	Postovulatory-phase ^b
1. Constituents of tubal secretion (μg)		
Total phospholipid	1,802	1,528
Protein	6,400	18,825
2. Extraction of phospholipids		
Total phospholipid (μg)	214.0	142.5
Phospholipid/person (μg)	44.0	25.6
PC/PL (%)	41.6	54.9
DSPC/PL (%)	16.9	12.9
DSPC/PC (%)	40.6	24.3
3. Composition of phospholipids (%)		
Phosphatidylcholine	41.6	54.9
Phosphatidylethanolamine	14.5	10.5
Phosphatidylglycerol	2.2	3.5
Sphingomyelin	21.0	18.1
Lisophospholipin	2.3	3.9
Others	18.4	9.1

^a Five persons/10 tubes.

^b Six persons/12 tubes.

two- to four-cell stage to develop further. We are of the opinion that Surfacten improves the culture medium to bring it more in line with the tubal environment. Since the advent of gamete intrafallopian transfer, cocultures, and other techniques of assisted reproductive technology, the importance of tubal physiology has become of interest because of its apparent functions in the maintenance of the zygote en route to the uterus. However, as the actual roles of the tubal endothelium and tubal secretions are as yet poorly understood, we began to study the surfactant-like material found in the tubal endothelium and lumen to elucidate its role in the maintenance and development of the zygote. We used ultrahistologic and histochemical approaches to localize this material. We also performed physiologic studies to determine its role in immunologic responses, sperm potentiation, and zygote development. We found that this material, used as an enhancing adjunct to the surgical achievement of tubal patency, as well as in in vitro fertilization (IVF) and embryo transfer (ET), may have beneficial effects. In vivo the ovum is fertilized in the ampullaris portion of the tube, undergoes cleavage while passing through the tube, and arrives in the uterine cavity as a blastocyst. Thus, one approach to improving IVF–ET success rates is to culture the zygote to the blastocyst stage before performing ET. At present, the common clinical procedure is to perform ET to the uterine cavity at the four- to eight-cell stage or at roughly 48 hr post-insemination. The reason for this is that prolonged culturing to obtain zygotes

beyond the eight-cell stage results in a high rate of degenerative changes.

The reason we selected surfactant as the material to be evaluated was that this material occurs naturally in the human female genital tract and responds in phase with the menstrual cycle. It is most abundant at or near the time of ovulation, when fertility is highest. This surfactant-like material was localized by techniques specific for pulmonary surfactant, including glutaraldehyde, paraaldehyde, and tannic acid fixation, and by observing the oviductal endothelium by transmission electron microscopy. We found surfactant-like granules in both the ciliated and the secretory cells of the tubal endothelium (Fig. 1). These granules are secreted into the tubal lumen by a merocrine process and in phase with the menstrual cycle, with increased abundance at the time of ovulation. These granules contain phosphatidylcholine, which binds with tannin as does pulmonary surfactant, suggesting strong effects in reducing surface tension. Surfactant specific apoprotein antibodies were used to evaluate immunohistologically the oviductal endothelium. The resultant reaction was stained with phosphin E, a surfactant-specific fluorescent dye. We found a strongly fluorescent reaction at the cell-lumen interface of the human oviductal endothelium, which was especially strong at ovulation at the tubal fimbria and isthmus. This material is highly abundant in the ampullaris, where fertilization normally occurs. It is also abundant in the uterine cervical mucus. We placed human spermatozoa in a pulmonary surfactant medium to investigate its capacitation of sperm in the form of the acrosomal reaction. The phenomenon was observed by scanning electron microscopy. We found sperm capacitation to occur at $60.4 \pm 19.6\%$, identified by the triple-staining method, for the surfactant-treated spermatozoa. In contrast, spermatozoa exposed to Ca^{2+} ionophore resulted in a $72.2 \pm 5.3\%$ capacitation rate. We then investigated the capacitation of spermatozoa by Surfacten with the hamster test. Surfacten-exposed spermatozoa were cultured with zona pellucida-stripped golden hamster ova to observe the penetration capacity of the ova by sperm. We found the tubal phospholipid and pulmonary surfactant to have equal results in the hamster test. Thus, this material also aids in the capacitation of the sperm and eases its penetration into the ova. It is also reported to assist zygote transport through the fallopian tube, as well as to provide a suitable environment for it to develop to the morula and blastocyst stage, when it enters the uterine cavity and undergoes nidation and further development. Our investigation was performed on bovine and hamster ova only, as

ethical considerations arise with the use of human ova. Our extrapolations to include the potential for improved success rates when used clinically to enhance fertility in women desiring to conceive, are based on the results of culturing bovine and hamster ova, and as our selected subjects were mammalian, perhaps the observations would be applicable to clinical practice. However, as stated previously, ethical considerations preclude clinical trials.

CONCLUSIONS

In human infertility management, complex media such as Hams F10 are being replaced by less complex media such as human tubal fluid. With the addition of surfactant, a constituent of tubal fluid, better-quality embryos which are more developed, perhaps even to the ideal of the blastocyst stage, at which the embryo normally enters the uterine cavity, may be obtained, leading to better pregnancy rates and more take home babies.

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