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Bovine In vitro fertilization: In vitro oocyte maturation and sperm capacitation with heparin

J.J. Parrish

Department of Animal Sciences, University of Wisconsin-Madison

1.0 Introduction

The report of in vitro fertilization (IVF) of bovine oocytes with frozen thawed semen and using heparin [1] has been important to most subsequent work with bovine IVF for research or the commercial production of embryos. The purpose of the experiments was to demonstrate that heparin was capable of increasing the ability of bovine sperm to fertilize bovine oocytes in vitro. The work was built upon research by others in the First and Ax laboratories at the University of Wisconsin as well as Bracket and coworkers at the University of Pennsylvania [2,3]. The review will begin with a discussion of the in vitro maturation procedures in 1986 and why results of IVF were reported differently than most researchers would recognize today. A discussion of the status of IVF and sperm capacitation in 1986, and how understanding heparin-induced capacitation now explains why other methods used to capacitate sperm in the 1980's likely succeeded follows. The final section will deal with current impacts of heparin and IVF in the in vitro production of embryos for research and commercial transfer. The review will not address culture conditions for embryo development as this was not part of the original publication [1].

2.0 In vitro maturation of oocytes

The oocyte maturation procedure used in Parrish et al., [1] and other publications associated with the First and Ax labs from 1983 to 1986 used a procedure with a Tyrode's base medium that was supplemented with fetal calf serum and a FSH preparation that had LH activity as described in Ball et al. [4]. While this succeeded in maturing oocytes in vitro to the stage at which oocytes were arrested at metaphase II of meiosis, it still had deficiencies. An ovulated oocyte would be at this same stage when penetrated by a sperm in the oviduct but would then be capable of forming both paternal and maternal pronuclei. Paternal refers to the sperm derived pronuclei and maternal as the oocyte derived pronuclei. This was not true of the in vitro oocyte maturation method described. To fully describe successful penetration, fertilization was expressed as penetration by sperm, 2-pronuclear formation and oocytes with evidence of penetration by only 1 sperm or a maternal pronuclei present. An example of one of the first oocytes fertilized by heparin-treated sperm in early 1984 is shown in Figure 1. The maturation of bovine oocytes under the conditions of Ball et al. [4] often resulted in reduced paternal pronuclei formation. Maternal pronuclei seemed to form if the oocytes were activated by sperm penetration. It would be found later that estrogen was

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Department of Animal Sciences, 1675 Observatory Drive, Madison, WI 53706, Phone: 608-263-4324; Fax 608-262-5157, parrish@ansci.wisc.edu.

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required and the Tyrode's based medium needed to be changed to a more complete cell culture medium, Medium 199 [2,5]. In addition, the gonadotropins FSH and LH, were now obtained from purified National Institute of Arthritis, Metabolism and Digestive Disease (NIAMDD) origin. These changes were sufficient for paternal pronuclear formation and supported full development [5–7] and the birth in 1986 of a calf from in vitro matured oocytes and in vitro fertilization. Rarely is failure of paternal pronuclei noted anymore with in vitro matured bovine oocytes. The key was most likely the inclusion of estrogen in the maturation medium. Many investigations by others were ongoing at the time using different serum supplements and coculture of oocytes during maturation with other cell types [2] but the basic method [5,6] is now standard with only modifications to source of gonadotropins.

3.0 Capacitation of sperm

Once oocytes are matured it is critical to expose those oocytes to sperm that have already been capacitated or are undergoing capacitation. Capacitated sperm have undergone biochemical modifications that allow them to acrosome react upon exposure to the zona pellucida, cumulus cells or other substances associated with in vitro matured or ovulated oocytes [8–11]. In the mid 1980's it was not always clear how specific sperm procedures impacted sperm to enhance IVF in the bovine. Effects could have been on capacitation, the acrosome reaction or both. If sperm were capacitating during incubation with oocytes, it was also important to consider if oocytes would age prior to sperm being capacitated and able to penetrate the zona pellucida. The source of sperm, ejaculated unfrozen or cryopreserved is also critical. One of the unique aspects of the Parrish et al. [1] work was the use of frozen-thawed semen as will be discussed later. However, using frozen-thawed semen results in many more sperm dying over incubation than would be seen with un-frozen semen. Such dead sperm complicate the interpretation of what is happening either before or during incubation with oocytes. Most of the works we will describe have used un-frozen semen for just this reason.

Bracket and coworkers in a series of reports [12–14] demonstrated that brief exposure of washed bovine semen to High Ionic Strength media (HIS) induced sufficient capacitation for sperm to fertilize in vivo matured bovine oocytes. The HIS medium was made by adding sufficient NaCl to the Bracket-Oliphant medium (BO; Table 1) to achieve 380 mosmols. The HIS and BO media had previously been shown to induce capacitation of rabbit sperm by presumably displacing decapacitation factors from the surface of sperm [15]. The results of treating either fresh or frozen-thawed bovine sperm with HIS treatment for IVF produced only modest penetration of oocytes by sperm. It was difficult to replicate this work and many results may have been dependent on the use of semen from particular bulls. A further limitation may have been that sperm were not sufficiently capacitated.

A different approach for capacitating bovine semen was also being developed by Ax and coworkers [4,16] and related to the possible role that follicular fluid and/or oviduct secretions play in capacitating or inducing the acrosome reaction in sperm. Follicular fluid and oviduct secretions are rich in glycosaminoglycans (GAGs) [17] and sperm were able to undergo the acrosome reaction after exposure to these compounds [4,16]. However Parrish et al. [18] was unable to demonstrate effects of the GAG, Chondroitin Sulfate A, in its ability to stimulate either acrosome reactions or fertilization frequencies. Heparin was found to stimulate both the acrosome reaction and fertilization but an interaction with the presence of glucose in the media was noted. Confusion had arisen from the description of the Tyrode's medium used by the previous studies. In the hamster, where the medium was originally described, glucose was in the final formulation [19]. It was unclear if glucose was used in the Tyrode's medium that was described for use with bovine sperm and GAGs [4,16]. It was discovered that the Tyrode's medium was made in two different labs where

people had different backgrounds that influenced whether they included glucose or not in medium. It would later be found that glucose delayed capacitation of bovine sperm by heparin [18]. Researchers should be aware that simply referencing a media may not be sufficient if slight changes have been made during development of the medium and precise formulations should be listed to avoid confusions. Compositions of various media used in oocyte handling, sperm preparation and during in vitro fertilization are thus listed in Table 1. The handling of oocytes or embryos between incubations is done in TL-Hepes that has reduced bicarbonate and Hepes added to maintain pH in an air atmosphere. Incubation of un-frozen semen is done in Sp-TALP but requires gassing of media and tubes with 5%CO₂ in air as the medium contains 25 mM bicarbonate and only 10 mM Hepes [20]. An alternative is the Sp-TALP-H which can be used in an air atmosphere, has only 10 mM bicarbonate but 40 mM Hepes but requires 5 hours for sperm to capacitate with heparin due to reduced levels of bicarbonate [21]. The medium Sp-TALP-H also is capable of supporting fertilization of oocytes in an air atmosphere if needed (Parrish, personal observation). The fertilization medium used is Fert-TALP and requires incubation in a 5%CO₂ in air atmosphere [1].

At the time when HIS and then later heparin were being investigated for treatment of sperm to increase IVF results, others were utilizing long incubation periods [22,23], or addition of caffeine to BO medium and/or addition of calcium ionophore [24]. It was difficult at the time to understand how these different methodologies were enhancing the ability of sperm to fertilize oocytes.

From the experiments using heparin with bovine sperm it is possible to come to a general understanding of the intracellular events of capacitation in the bovine. The first observation of importance is that heparin induces capacitation of bovine sperm rather than the acrosome reaction. This is best demonstrated by the requirement of at least a 4 hr incubation with heparin before sperm can either undergo a stimulus induced acrosome reaction from lysophosphatidylcholine [20], soluble zona pellucida proteins [10,25], or penetrate zona intact bovine oocytes [20]. Heparin must first bind to bull sperm prior to its ability to induce capacitation [26,27] and the ability to capacitate resides in the charge dependent nature of this binding [26,28,29] as it can be inhibited by protamine sulfate [27]. The binding of heparin is to a series of bovine seminal plasma proteins (BSPs), that bind to epididymal sperm at ejaculation [30]. These proteins include BSP-A1, BSP-A2, BSP-A3 and BSP-30-kDa. The BSPs interact with both cholesterol and phospholipids in the sperm plasma membrane. Following heparin binding, there is a loss of lectin binding to bovine sperm indicating the loss of sperm surface components [31,32]. The changes in surface components likely relate to a heparin induced loss of the BSPs over time that lead to a loss of membrane cholesterol and phospholipid [30]. Discussions of the role of cholesterol loss and membrane modifications are discussed in Bailey [33], Leahy and Gadella [34] and Gadella [35].

As the loss of BSPs must be occurring, changes to sperm intracellular pH (pH_i), intracellular calcium (Ca_i) and cyclic adenosine 3'5'-monophosphate (cAMP) levels also happen due to heparin. Investigations into the role of the intracellular changes during capacitation of bovine sperm induced by heparin have been greatly helped by the effects of glucose. An interaction of heparin and glucose on sperm capacitation were first noted in Parrish et al. [18]. The effect of glucose is not on heparin binding to sperm as heparin binding was not affected by the presence of glucose [27]. Glycolysis of glucose or other similar substrates leads to an acidification of bovine sperm that blocks heparin-induced capacitation [21]. The major effect of glycolysis is that proton (H⁺) production acidifies the pH_i of sperm, which opposes the heparin-induced alkalinization of pH_i [21,36]. The effect of glucose can be circumvented by addition of compounds that increase intracellular cAMP such as a 8-bromo-

cAMP, isobutylmethylxanthine (IBMX), and caffeine or allowing sufficient time for sperm to metabolize all the glucose present [18,27,37]. Ability of sperm to metabolize a substrate under closed incubation conditions has rarely been taken into account during capacitation or fertilization experiments. A rise in sperm cAMP is needed for heparin-induced capacitation [27] but blocking the effects of cAMP with a specific inhibitor such as RP-cAMP does not prevent the increase in pH_i [37] suggesting the cAMP increase is downstream of the change in pH_i . Heparin thus binds to sperm, BSPs are lost along with membrane cholesterol, pH_i increases and then cAMP increases.

Calcium is important to capacitation of sperm. Ejaculated bovine sperm have a very active plasma membrane calcium ATPase that extrudes calcium and maintains Ca_i in the nM range. Capacitation of bovine sperm with heparin requires extracellular calcium that is taken up by sperm and leads to a rise in Ca_i in the sperm head [38,39]. Glucose blocks the uptake in calcium but this can be overcome by the addition of cAMP modulators that increase cellular cAMP [38]. Interestingly, as heparin induces calcium uptake by sperm, initially Ca_i in the head is low at 102 ± 13 nM but then increases to 184 ± 21 nM by 4 hr of incubation, when sperm are capacitated [40]. Evidence suggests that calcium uptake is critical for capacitation during the first 2 hours of heparin exposure [39]. During this time, the acrosome accumulates calcium and thus prevents a Ca_i increase in the cytoplasm. As the acrosome store fills, Ca_i then increases and if a sperm does not come in contact with the appropriate stimulus, a spontaneous acrosome reaction and sperm death occur. The appropriate physiological stimulus would be the zona pellucida [10,25]. During the acrosome reaction, the internal store would be released and a store-operated plasma membrane calcium channel appears to be activated to increase calcium in the sperm cytoplasm [39]. The final increase in Ca_i is important and is related to the physiological ability to acrosome react. This was demonstrated in an experiment in which bovine sperm were loaded with Fura2 to measure Ca_i and then incubated with and without heparin [41]. At 5 hours of incubation, sperm were imaged for Ca_i in the sperm head and then exposed to soluble zona pellucida proteins and monitored for an additional 15 minutes to determine if they acrosome reacted. Control, uncapacitated sperm that did not or did acrosome react had 61 ± 3 ($n=207$) and 78 ± 7 ($n=7$) nM Ca_i in the sperm head and were not different, $p>0.05$. In comparison, sperm incubated with heparin and did not or did acrosome react had 102 ± 9 ($n=97$) and 311 ± 42 ($n=58$) nM Ca_i in the sperm head ($p<0.05$). Of the heparin treated sperm that acrosome reacted, the higher the Ca_i , the quicker sperm acrosome reacted in response to zona proteins. Examination of the anterior and posterior head Ca_i found evidence that only sperm with Ca_i increases in the acrosome actually underwent a zona pellucida induced acrosome reaction.

Two other observations on heparin-induced capacitation are important [29]. The first is that a minimum of 10 mM bicarbonate is required in capacitation or fertilization medium. Sperm contain a soluble adenylate cyclase, present in the cytoplasm that is stimulated by bicarbonate. The increase in sperm cAMP during capacitation likely does not occur in the absence of bicarbonate. The second observation is that bovine serum albumin (BSA) is almost always present in capacitation and fertilization media. Heparin-induced capacitation does not require BSA, but BSA is required for capacitated bovine sperm to undergo an acrosome reaction either in response to lysophosphatidylcholine or zona intact oocytes. Heparin likely leads to cholesterol loss from sperm via the interaction with BSPs and so a potential cholesterol acceptor like BSA is not needed to remove cholesterol from sperm and modulate the dynamics of the plasma membrane. The exact role of BSA during the acrosome reaction remains unclear.

The question is often asked if heparin capacitates bovine sperm in vivo? Bovine oviduct fluid can capacitate bovine sperm in vitro in a time course similar to heparin and an active

component is a GAG similar to heparin, likely heparan sulfate [27,42]. The GAGs in the oviduct likely reside on oviduct epithelial cells as proteoglycans and interact with sperm upon their binding to these cells in vivo. Interestingly bovine oviduct fluid has low levels of glucose [42]. However oviduct fluid does not increase sperm cAMP and so differences with heparin exist that may be explained by multiple capacitation pathways activated by heparin in vitro [27]. The discovery of other protein capacitating agents in bovine oviduct fluid [43] suggests that GAGs might not be the only agents responsible for capacitation. Heparin still capacitates bovine sperm in vitro and is a major asset to IVF in the bovine.

Following the changes in sperm pH_i , Ca_i and cAMP, there is an activation of protein tyrosine kinases and potentially inhibition of protein tyrosine phosphatases [33]. The changes modulate sperm to be able to undergo an acrosome reaction when encountering the zona pellucida of the oocyte. A model that encompasses everything noted above in the effects of heparin on bovine sperm is reflected in the model of capacitation in Figure 2.

If we use the knowledge gained by examining how heparin capacitates bovine sperm, the other methods for capacitating bovine sperm that were developed in the late 1970's through the 1980's can be explained. The methods that used the HIS medium likely were displacing BSP's from sperm thus decreasing membrane cholesterol and altering membrane biophysical properties. The BO medium contained glucose and so was working in opposition to the HIS effects and likely delayed or prevented capacitation in many males. Long incubation periods likely also involved the gradual loss of BSPs and associated membrane cholesterol. Male variation in affinity of the BSPs would likely have been important in finding a male whose sperm would capacitate before motility and viability of sperm decreased. The BO medium with addition of caffeine and/or a calcium ionophore mimics the need for an increase in cAMP and Ca_i during bovine sperm capacitation [24]. However the used of BO medium and cAMP modulation works better when heparin is also included in the procedure [44,45]. The large number of publications related to bovine IVF since 2000 make it impossible to exhaustively examine procedures. However it is possible to make a general conclusion. There are 2 procedures that stand out and both use heparin. The first is the BO medium with cAMP, and heparin treatment. The second is modifications of the 1986 heparin paper [1] where heparin is simply added to the fertilization medium in different amounts along with sperm washing via a Percoll or similar gradient [2,46].

4.0 Use of cryopreserved semen for IVF and adjustments for bull effects

Frozen-thawed semen was used in Parrish et al. [1] and was critical to repeatable production of in vitro produced (IVP) embryos. The sperm treatment procedure was adapted from that used on ejaculated sperm [18] in which sperm were pre-incubated with heparin. Thus sperm were incubated 15 minutes with 10 μ g/ml heparin and then diluted into the fertilization medium containing in vitro matured oocytes. The short incubation time is due to frozen-thawed semen having reduced ability to survive incubation when compared with unfrozen semen. This allowed a carryover of 0.2 μ g/ml heparin during fertilization. It was the carryover level of heparin that was critical and we later showed that fertilization rates with frozen-thawed sperm were heparin dose dependent [47]. Following this discovery, heparin was added directly to the fertilization medium. Using this approach, most oocytes are penetrated by sperm within 4–6 hours with pronuclei forming between 6 – 10 hours after sperm addition [7,48]. It is possible then to adjust the percentage of oocytes fertilized by adjusting heparin levels. Generally these have ranged from 0.2 to 5 μ g/ml but higher levels can be used. Changing the amount of sperm added to the bovine in vitro fertilization system also impacts the fertilization [47,49]. From years of experience, the rate of fertilization of in vitro mature oocytes is associated with rates of polyspermy [49]. When fertilization rates exceed 80%, polyspermy begins to increase, thus impacting eventual development rates. The

heparin system with frozen-thawed semen gives two critical points at which to adjust fertilization rate for a particular bull. You can change heparin levels and/or you can change sperm concentrations to get optimal fertilization results that maximize sperm penetration of oocytes but minimizes polyspermy. Because many straws can be frozen from a single bull ejaculate it is then possible to fertilize oocytes with the same preparation of sperm and eliminate the male to male or ejaculate to ejaculate variability.

5.0 Sperm washing procedures

Sperm preparation for IVF generally involves some procedure to separate spermatozoa from seminal plasma, extender and/or cryoprotectants. As pointed out above, the number of sperm added to oocytes during IVF impacts the percentage of oocytes penetrated by sperm and even penetration by multiple sperm in polyspermy. Semen contains both motile or viable sperm as well as non-motile sperm that are usually dead and will not be capable of fertilizing oocytes. In order to get repeatable results with IVF it is essential to add the same number of sperm that have the potential to participate in fertilization. With unfrozen semen, most sperm are motile/viable so simply determining the concentration of sperm and adding a set amount is sufficient. While frozen-thawed sperm provide the advantage of using semen from the same bull and even ejaculate, many sperm die in the cryopreservation process resulting in post-thaw motilities of 30 – 70% [46]. Swim-up procedures were adapted to circumvent this problem [1]. In swim-up, sperm are layered at the bottom of a column of medium. The dense nature of semen in extender and cryoprotectant initially keeps these sperm at the bottom. Over time sperm begin to swim-up out of the extender and cryoprotectant into the covering medium. Isolating just the covering medium provides a population of sperm that is close to 100% motile or viable. This swim-up isolated population of sperm can simply be counted in order to add a specific number of sperm to the IVF system. While simple in principle, the swim-up technique was difficult for many to implement. For example, if you get extender associated with the isolated sperm, the extender inhibits capacitation and so fertilization. Further semen frozen for IVF use was often at 75 to $100 \times 10^6/\text{ml}$ and much more than the $15 - 30 \times 10^6/\text{ml}$ used for commercial artificial insemination. The number of sperm recovered from swim-up with commercial semen was then often quite low. A Percoll gradient system similar to that used with human sperm was optimized for bovine semen between 1989 and 1990 and shared with many labs for use in preparing bovine sperm for IVF. A comparison of the Percoll approach for sperm isolation with the swim-up method was described in Parrish et al. [46]. Recovery of motile sperm from frozen thawed semen was $9\% \pm 1$ for swim-up approach but $40\% \pm 4$ for Percoll. The higher recovery is why Percoll or something similar has been generally adopted. It was noted that at the same sperm numbers, IVF rates were higher for the swim-up isolated sperm. Increasing the sperm concentration during fertilization however easily compensated for this deficit in Percoll separated sperm.

6.0 Current status of IVF and embryo production

The IVF work done in the bovine has been valuable to both the scientific and commercial interests. The work of Parrish et al. [1] now has greater than 800 citations, based a search of the Web of Knowledge/Web of Science. The bovine is one of the best models to study in vitro embryo development. A search using Pub Med found over 1000 publications related to bovine embryos from 2011 – present. The large supply of bovine oocytes obtained from either slaughterhouse material or ovum pick-up procedures with ultrasound guided follicular aspiration makes it possible to produce embryos in any quantity desired. This is best illustrated by the worldwide statistics on IVP embryo production and transfer in 2011 [50]. There were 453,471 IVP embryos produced and 343,927 transferred, the majority of which are in Brazil. While it is not possible to track down procedures used for IVF in all the

laboratories involved, most appear to be using some sort of procedure that involves heparin. The number of IVP transferred embryos has been steadily increasing since 2001 and is fast approaching the in vivo produced and transferred embryos of 572,342.

7.0 Concluding remarks

The development of the IVF system in the bovine did not occur in a vacuum. The majority of the work was done in the laboratory of NL First but interaction with the lab of RL Ax also occurred. The NL First lab had been assembled in the 1980's to develop cloning and gene modification methodology in the bovine but individuals all were working in solving problems in specific areas. Many of the names will be recognized by researchers of today and include (listed in alphabetical order): FL Barnes, ES Critser, WH Eyestone, HM Florman, RR Handrow, ML Leibfried-Rutledge, DL Northey, JJ Parrish, RS Prather, JM Robl, CF Rosenkrans, ML Sims, MA Sirard, JL Susko-Parrish, CB Ware, MA Winer. The unique nature of the individuals and stimulating intellectual environment made the developments for the bovine IVF system possible along with many other contributions to science.

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Figure 1.

In vitro matured and fertilized bovine oocyte. The oocyte was one the first matured in vitro and fertilized with heparin-treated sperm in early 1984 as described in Parrish et al. [1]. Two pronuclei (PN) are shown along with the tail of the penetrating sperm (ST).

Figure 2.

Proposed model of intracellular events during capacitation of bovine sperm by heparin. Ejaculated sperm bind seminal plasma proteins (BSP) at ejaculation. Heparin used during in vitro fertilization (IVF), binds to BSP and leads to their loss from the plasma membrane along with associated cholesterol and phospholipids. Other cholesterol acceptors in the capacitation/fertilization medium such as BSA are present and may also absorb membrane cholesterol. This leads to changes in the plasma membrane [35]. Intracellular events are then related to a heparin-induced decreased ability of sperm to extrude Ca²⁺ via a calcium-ATPase. A net uptake of calcium occurs and at first Ca²⁺ is taken up by the acrosome and intracellular Ca²⁺ (Ca_i) does not change until this store is filled. Once the acrosome is filled with Ca²⁺, Ca_i begins to increase. Heparin binding also induces a net efflux of H⁺ and presumed influx of HCO₃⁻. The model does not assume the changes in HCO₃⁻ and H⁺ are linked through for example a dual transporter. The net changes in HCO₃⁻ and H⁺ increase intracellular pH (pH_i). Sperm soluble adenylate cyclase (sAC) is stimulated by both HCO₃⁻ and increasing pH_i. The resulting cAMP activates protein Kinase A (PKA) and through cross talk, protein tyrosine kinases (PTK) are stimulated and protein tyrosine phosphatases (Ptyr-Ptase) are inhibited. A net increase in protein tyrosine phosphorylation thus occurs. The rising Ca_i further stimulates sAC and the inhibition of Ptyr-Ptases. The capacitated sperm is thus primed to undergo a zona pellucida induced acrosome reaction.

Table 1

Common media used for capacitation and fertilization of bovine gametes.^a

Media	BO ^b	Sp-TALP ^c	Sp-TALP-H ^d	TL-Hepes ^e	Fert-TALP ^f
Component	Units				
NaCl	112.00	100.00	87.00	100.00	114.00
KCl	4.02	3.10	3.10	3.10	3.20
CaCl ₂	2.25	2.00	2.00	2.00	2.00
NaH ₂ PO ₄	0.83	0.30	0.30	0.30	0.30
MgCl ₂	0.52	0.40	0.40	0.50	0.50
NaHCO ₃ ⁻	37.00	25.00	10.00	2.00	25.00
Hepes	-----	10.00	40.00	10.00	-----
Glucose	13.90	0	0	0	0
Pyruvic acid	1.25	1.00	1.00	0.20	0.20
Lactic acid	-----	21.60	21.60	10.00	10.00
BSA	10	6	6	3	6
Penicillamine	0-20	-----	-----	-----	20
Hypotaurnine	0-10	-----	-----	-----	10
Epinephrine	0-1	-----	-----	-----	1
Sodium Metabisulfite	?	-----	-----	-----	2

^aFormulations were from several publications [3,20,21,36]. Most media utilize some type of antibiotic such as 50 µg/ml Gentamycin.

^bBracket and Oliphant medium [15].

^cStands for sperm TALP (Tyrode's medium base, albumin, lactate and pyruvate). Also known as bovine gamete medium 1 (BGM1) in Parrish lab publications. Must be incubated under 5% CO₂ in air to maintain pH.

^dThe H is for high amount of Hepes. This medium should be incubated in air and will support IVF under an air atmosphere if desired. This medium is as known as BGM3 in Parrish lab publications.

^eMedium used to wash oocytes prior to IVF.

^fFertilization TALP as described in Parrish et al. [1]. The medium must be kept under a 5%CO₂ in air atmosphere to maintain pH.

? - It is not known if sodium metabisulfite was present but it is used to stabilize the Penicillamine, Hypotaurnine and Epinephrine preparation and directly improves IVF results.