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# In Vitro Fertilization Studies On *In Vitro* Matured Cattle Oocytes Using Heparin,Epinephrine And Pencillamine In The Culture Medium

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***Abstract:*** *Sire effect on **in vitro** fertilization on **in vitro** matured cattle oocytes were tested using frozen thawed semen. Oocytes were collected from the ovaries of slaughtered cattles. Follicular fluid were aspirated and screened for good quality oocytes. It is then washed and cultured in the maturation media. After incubation at $38^oC$ for 12-15 hours in a $CO_2$ incubator it is fertilized with washed semen in the fertilization medium. It is again incubated at $38^o$ C for 12-15 hours keeping in a $CO_2$ incubator. Oocytes are then taken out washed, fixed, stained and observed for fertilization. Fertilization rate for different bulls were calculated. Semen from different bulls found to have different rate of fertilization on **in vitro** matured oocytes. The fertilized embryo can be transferred to embryo culture medium for further development to **blastocyst** and **stem cell** production.*

***Key words:*** ***In vitro fertilization, Capacitation, Acrosome reaction, Stemcell, Cloning***

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## I. Introduction:

Fertilization is a complex process involving a number of cellular changes. The fertilized egg develops to a ***blastocyst*** and the ***stem cells*** present in the blastocyst can differentiate into specific organ tissues. Extensive studies were carried out to culture, fertilize the oocytes in vitro and to develop the embryo in culture. This is known as IVF technology. The revolutionary potential of this technology lays in the production of ***stem cells***. Stem cells can be used for the treatment of a number of incurable diseases like *Parkinson's, Neuron degenerative, Diabetes, Heart tissue replacement* etc. It is also useful in ***cloning*** and gene microinjection for the production of ***transgenic*** animals.

A large number of experiments were conducted for in vitro standardization of oocyte maturation, sperm capacitation, fertilization, and embryo development in different species. There are different factors like concentration of hormones, serum, capacitating agents etc.which effects the fertilization rate of the spermatozoa. Sire or the bull effect is one among the factors, which effect the fertilization rate of ***in vitro*** matured cattle oocyte.. ***In vitro*** maturation of oocytes were done successfully in a number of mammalian species like Mouse (Choi T.S.et al., 1987),Sheep (Cheng et al., 1986),Rabbit (Chang M.C.et al).,.The oocytes maturation studies were extensively carried out in buffalo by (Totey et.al., 1992)

Before fertilization the spermatozoa undergoes various biochemical changes like capacitation and acrosomal reaction. Fertilization is cellular process which is controlled by different factors like concentration of capacitating agents.$Ca^{++}$ ionophores, caffeine, heparin etc.can be used as capacitating agents. (Ball et al.,1994), (Parrish J.J et al.,1984).Different studies established the fact that sperm from different males within a species varies widely in their ability to fertilize the oocyte. Capacitation which is a cellular process is being effected by different concentration and combination of heparin,calcium ionophores, and different culture media. (Parrish et al., 1985);(Leibfried et al.,1987) and (Jiang et al., 1991). The variation among males is even apparent when using an equal number of highly motile sperm. (Parrish et al.,1986).

The effect of heparin to capacitate the sperm is depending on the number of heparin binding sites on the sperms.( Marks and AX et al.,1986).This may vary from bull to bull.

The bull effect on the ***in vitro*** fertilization on cattle oocyte has not been studied yet especially in India and main objective of the study is to establish the **bull effect/sire effect** and the *heparin optimization for each bull*.

## II. Materials and Methods

All chemicals were purchased from **Jain biologicals**, Mumbai, India

10 ml of maturation media was prepared as Ham's F-10-9 ml, FCS-1 ml. Na Pyruvate-100 µl. Gentamycin-10µ L,LH-10 µg/ml , FSH-0.5 µg/ml, filter sterilize and Estradiol of 1 µm/ml were added (Totey et al.,1992) .Maturation plates were prepared by making 100µl of droplets of media in 60mm petri dish. Drops were covered with 100ml of sterile paraffin oil. It is then equilibrated in $CO_2$ incubator at 38°C.

Small dishes with maturation medium were also kept in $CO_2$ incubator to give a final wash to the cumulus oocyte complex. TL Hepes was prepared (Totey et al.,1992) for washing the matured oocytes which was also kept in $CO_2$ incubator for equilibration.

Oocytes were collected from cattle ovaries obtained from the slaughter house daily. It was brought to the laboratory within 2-3 hrs. of slaughter in a thermo flasks with warm water at 30-35 $^0$c supplemented with 5ml of gentamycin. Ovaries were thoroughly washed five times with warm water. Follicle with 2-6 mm diameter was then aspirated using 10ml syringe and 18-gauge needle. The follicular fluid was collected in 50ml sterile conical tube and kept at 38 $^o$ C for 10-15 minutes to settle down the debris. Then spin at 500 rpm for 5 min. The supernatant was then discarded and the pellet was carefully diluted with TL medium buffered with10 mm HEPES and low bicarbonate TALP (Parrish et al., 1986) which was pre incubated at 38°C in $CO_2$ incubator for 4-5 hrs. It is then screened under microscope for oocytes. The mature oocytes are having compact cumulus cells. The nude ones are discarded. The good oocytes are also having clear cytoplasam.

The collected oocytes are then throughly washed in TL- HEPES and then in maturation medium. It is then kept in maturation drops and layered with paraffin oil which was kept in $co_2$ incubator for equilibration.This oocytes were again kept in $CO_2$ incubator at 38°C for 15-18 hrs for maturation. (Totey et al.,1992). Fully matured oocytes with intact cumulus cells were collected and taken for fertilization treatment.

Fertilization media consists of Modified TL medium with bicarbonate, Heparin Fatty acid free BSA. (Downs S.M.et al.,1984).(Item II.4) The concentration of heparin varies from bull to bull. PHE is also added if required. It is then filtered and 50 µl drops were made in 30 mm sterile petri dish and layered with sterile paraffin oil. Dishes were then kept for pre incubation at 38°C in a $CO_2$ incubator.

One straw of semen of a particular bull is taken out from liquid nitrogen and kept at 39$^O$ C in a water bath. Dead and live sperm were separated by percoll separation (Fukui et al., 1983). 2 ml of 90% Percoll were layered by 45 % percoll and pre incubated at 38$^O$ C. The sperms were layered on it and spinned at 2000 rpm for 30 minutes. The sperms will be separated by density gradient. Live sperms will be in 90% percoll. Live sperms were collected and washed with sperm washing medium spin at 2000 rpm for 30 minutes. and the supernatant was discarded. The pellet is then diluted to a required concentration of 2 x $10^6$ sperms /ml with SPTL . (Fuki et al 1990),(Niwa et al ¨1988). It is then incubated with 10 oocytes /50µ l of the fertilization drops. The fully matured oocytes were collected from maturation media,washed with fertilization media and kept in fertilization drops i.e. 10 oocytes/50µl of fertilization drop.Then washed diluted semen is added and kept for fertilization at 38°C in a $CO_2$ incubator for 15 to 18 hrs.Fertilized oocyte is either used for fixing or transferred to embryo development medium.

Depending on the bull the concentration of pencillamine, epinephrine,and heparin varies. These are prepared as in stocks and kept at 4°C. (Niwa et al., 1988). Heparin is added in different concentration depending on the bull.

Frozen thawed semen collected from Kerala Live Stocks department were used for **in vitro** fertilization. The semen was incubated with oocytes for 15-18 hrs in $CO_2$ incubator at 38$^O$C .The oocytes were then taken and the cumulus cell were removed by repeated pippeting with a small bored pipette. Cumulus free oocytes were either mounted or transferred for embryo development. It is fixed on a glass slide with 3:2 alcohol and acetic acid in the ratio 3:2. as fixative , keeping at room temperature for 18-24 hrs. and stained with 1% orcein in glacial acetic acid. Acetic acid dissolves zona pellucida. It is then visualized using phase contrast microscope (10x). Oocytes were classified as fertilized and not fertilized. Ferilized oocytes were having two pronucleus. One male pronucleus with associated tale are visible within the oocyte having a second pronucleus or chromosome in either **anaphase II** or **telophase II** or **metaphase II of meiosis**. Oocytes were classified as normal if it is fertilized for single sperm otherwise poly spermy if fertilized by multiple sperms. If there is contamination the oocytes get degenerated.

**Statistical analysis**.

The fertilization rate was observed for different heparin concentration of same bull and also for different bulls. The same experiment is repeated in replicate and data was analysed by **2 x 2 chi square** test.

## III. Results

The normal fertilization rate was significantly higher for spermatozoa treated with 10 µl/ml of Heparin compared with 1µ l /ml of heparin.For the bull no.1, a maximum fertilization rate of 36.1 % were obtained with 10µl/ml heparin .(**Fig.1**)

The spermatozoa treated with **10 µ l /ml** of heparin gave significantly higher fertilization rate than without treatment of Heparin.The fertilization rate decreased as the concentration of heparin increase to 100 µl /ml. The maximum fertilization rate obtained was **28.2 %.(Fig 2)**

Increasing of Heparin concentration increased the fertilization rate significantly compared to that without heparin. When heparin rate was increased to 1-10µl /ml the fertilization rate increased significantly. But the rate decrease d as the heparin concentration increased from 10 to 100µ l/ml Bull no 3 give a maximum rate of **31 %** fertilization with 10 µl/ml of heparin and (**Fig 3)**

In the present experiment the spermatozoa of bull no 4 were treated with different concentration of heparin .No significant difference were observed for normal fertilization rate. The maximum normal fertilization was found to be **10.75%** for a heparin concentration of **10 µl/ml.(Fig.4)**

A maximum of **52%**fertilization was observed for bull no. 5, when the spermatozoa is treated with **10µ l /ml** of heparin**. (Fig.5)**

In conclusion the spermatozoa treated with different heparin concentration showed different rate of fertilization. and each bull has an optimum level of heparin for obtaining a maximum rate of fertilization. The results were summarized in the **table no. I**

The fertilization rate varies from bull to bull. (Fig.6).The maximum fertilization rate of **52** % was obtained for the bull **no. 5** and a minimum of **10.8%** for bull **no.4** were observed. The maximum rate of fertilization were 36.1 **%** for bull no.1, 28.2 **%** for bull no.2, 31% for bull no.3.

## IV. Discussion

The potential of **IVF** technology for improving genetic stock of domestic animals has been widely realized. This technology can be effectively used for the production of embryos of desired quality in large number. It has been realized that the blastocyst is a good source of ***stem cells*** which can differentiate into any kind of organ tissue. This fact directs ***IVF*** technology to a new field of tissue engineering and ***stem cell*** research. Thus rate of fertilization and embryo production have significant importance in ***stem cell*** programmes..

Austin et al., (1951)[17] have proved that mammalian ejaculate of sperm does not undergo fertilization directly .It has to undergo a cellular reaction called capacitation before fertilization, which occurs, in female reproductive tract. Parrish et al .,(1985)[7] showed that heparin sulphate is most potent glycosaminoglycan and can cause sperm capacitation in a dose dependent manner. This fact is again established in this study.

In the present investigation frozen thawed semen of five bulls were used. The fertilization rate was variable among the tested bulls (Fig no.6).Bull no.5 and 1 showed maximum fertilization rate of 52 % and 36.1% at the heparin concentration of 10 µ l/ml.(fig.1&5), than that of 0 and 100µ l/ml. of heparin concentration .Bull no.2 and 3 showed a maximum fertilization rate of 28.2 % and 31 % respectively at heparin concentration of 10µ l/ml.(fig 2&3). The bull no.4 (fig 4) was observed the lowest fertilization rate of 10.75%.The effect of individual bull and heparin concentration on the fertilization rate of ***in vitro*** matured cattle oocytes in this study confirms the findings of the report on other mammals (Parrish et al.,1985a).(Leibfrierd et al.,1989),(Moor et al.,1988),(Totey et al.,1993).From the present observation we found that each bull required different concentration of heparin to achieve maximum fertilization rate. Parrish et al.,(1988) reported that the ability of heparin to capacitate the sperm depends on the binding sites on the sperm surface.This number varies among bulls.(Marks and Ax 1985) showed that increased dosage of heparin had increased the penetration.(Totey et al .,1993) reported a decrease in penetration rate with increase of heparin concentration.

Difference among bulls in the success of in vitro fertilization rate was observed in several different system of capacitation including various combinations of caffeine, hypotaurin, epinephrine (Niwa et al., 1988).The biology and chemistry of fertilization ( Wassarman et al.,1987) has yet to be analyzed for mammals keeping a view on its effect on **stem cell** formation.

Studies are yet to be carried out in cattle oocytes to determine the presence of GAG's in follicular fluid, oviductal fluid or cumulus cells. Since heparin induces the capacitation rate in cattle oocyte it is interesting to study the nature of GAG's present in utrine fluid. **The media composition for maturation, fertilization, development has a great effect on *embryonic stem cell* production and further differentiation into organ tissue. This has to be analyzed and studied extensively** This study may help in the successful production of ***embryonic stem cell*** in cattle.

**In conclusion heparin capacitate bull spermatozoa in a dose dependent manner and the fertilization rate varies from bull to bull.**

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**TABLE NO:I**

| BULL NO | HEPARIN CON µl/ml | PHE CONCENTRATION µl/ml | No of oocytes cultured | No of oocytes fertilized | FERTILIZATION RATE % |
|---|---|---|---|---|---|
| **1** | 1 | 1 | 120 | 3 | 2.5 |
| | 5 | 5 | 100 | 13 | 13 |
| | 10 | 10 | 125 | 45 | **36.1** |
| | 100 | 10 | 100 | 23 | 23 |
| | 10 | 100 | 100 | 15 | 15 |
| **2** | 1 | 1 | 100 | 9 | 9 |
| | 5 | 5 | 105 | 15 | 14.1 |
| | 10 | 10 | 103 | 29 | **28.2** |
| | 100 | 10 | 103 | 24 | 23.1 |
| | 10 | 100 | 110 | 14 | 12.8 |
| **3** | 1 | 1 | 105 | 24 | 24.7 |
| | 5 | 5 | 100 | 33 | 33 |
| | 10 | 10 | 100 | 31 | **31** |
| | 100 | 10 | 100 | 32 | 32 |
| | 100 | 100 | 102 | 24 | 23.6 |
| **4** | 1 | 1 | 108 | 9 | 8.2 |
| | 5 | 5 | 105 | 14 | 13.1 |
| | 10 | 10 | 110 | 12 | **10.75** |
| | 100 | 10 | 107 | 35 | 32.9 |
| | 100 | 100 | 106 | 30 | 28.2 |
| **5** | 1 | 1 | 100 | 10 | 10 |
| | 5 | 5 | 102 | 23 | 22.6 |
| | 10 | 10 | 100 | 52 | **52** |
| | 10 | 10 | 102 | 25 | 24.9 |
| | 100 | 100 | 128 | 26 | 20.2 |

**FIG 1**

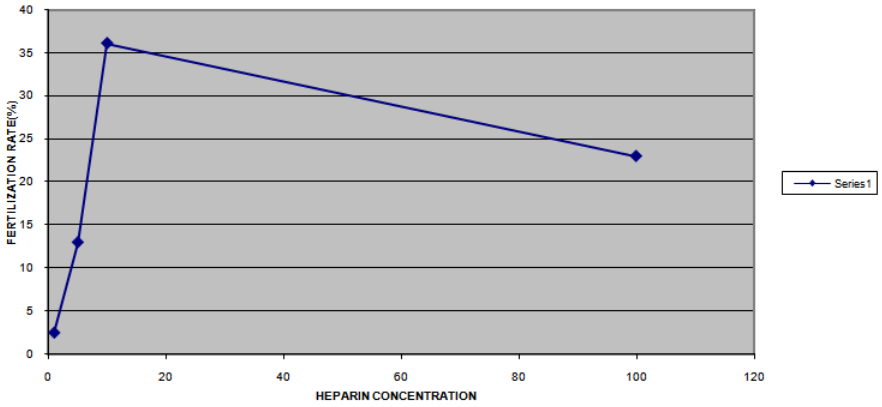



**Fig 2**

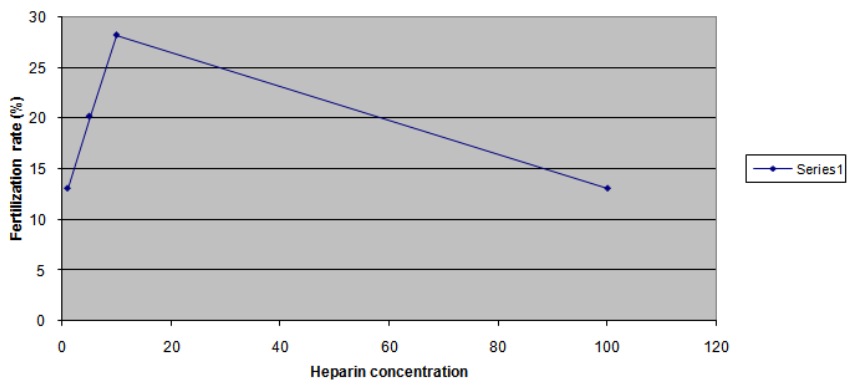


**Fig 3**

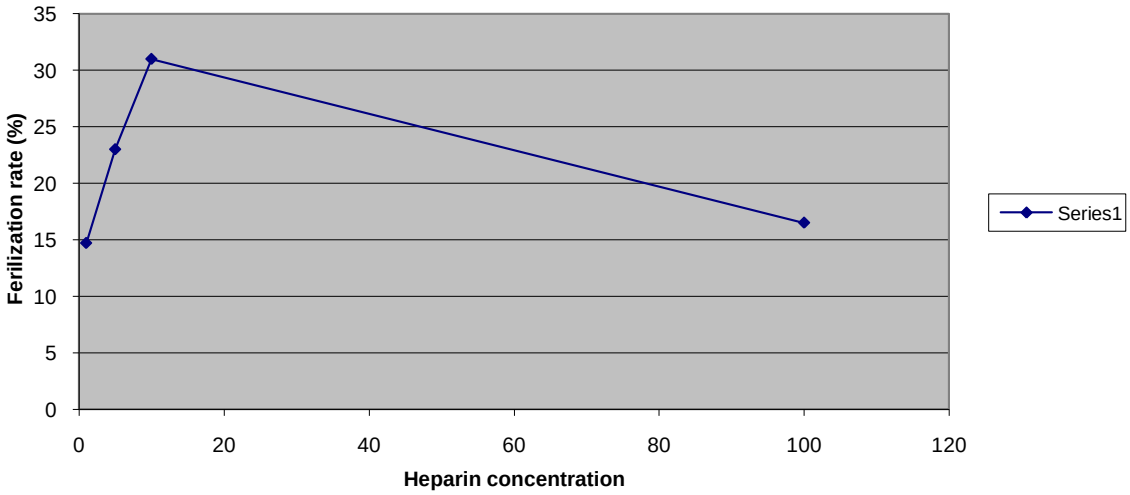



**Fig 4**

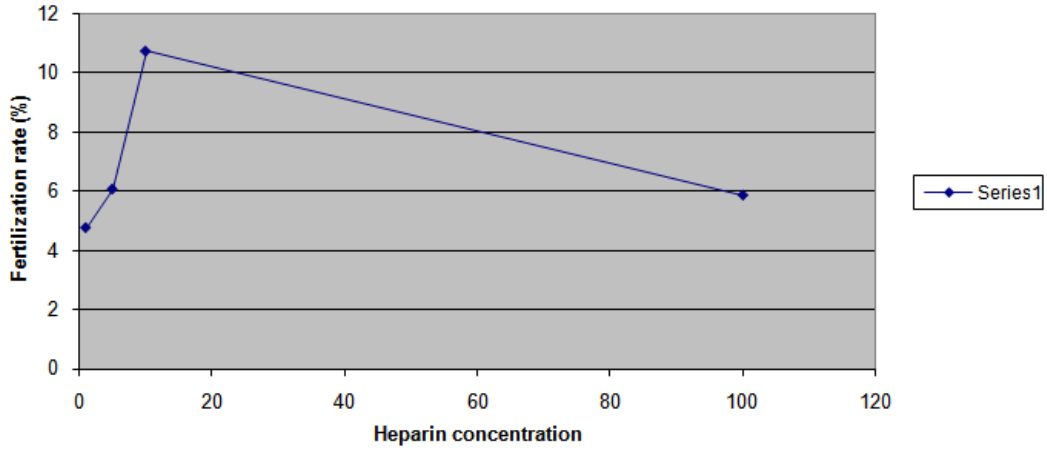



**Fig 5**

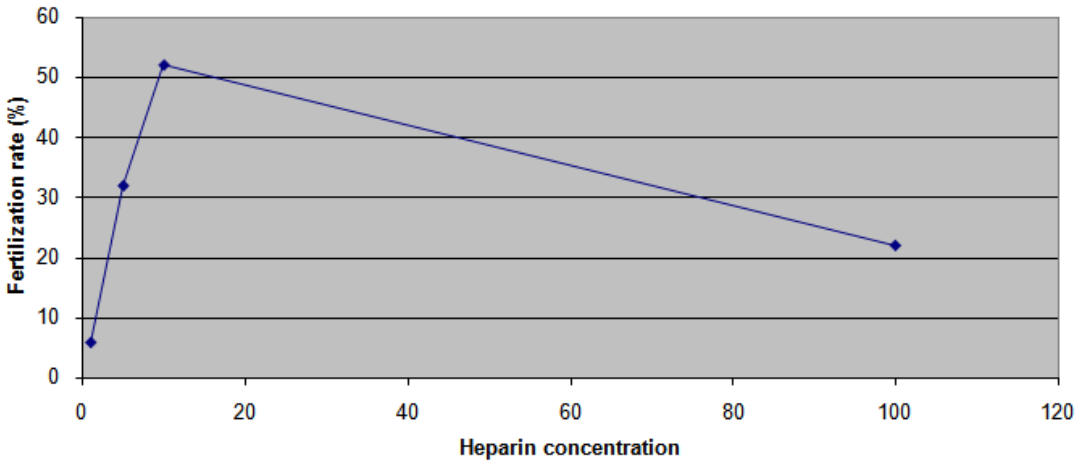
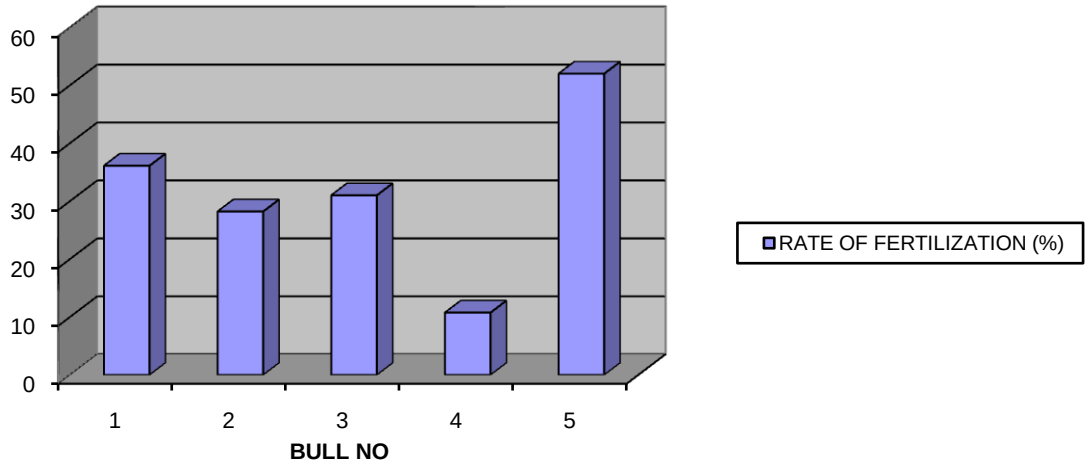


**Fig 6**

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