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Preparation of progesterone nanoparticles and evaluation of its effect on the capacitation of Bovine spermatozoa used in the *in Vitro* Fertilization

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Abstract

Progesterone (P) has been reported to affect several sperm functions especially capacitation and acrosome reaction. The main problem of (P) is its low aqueous solubility. So formulation of progesterone nanoparticles (PN) will enhance its solubility. This study was conducted to produce nanosized progesterone (NP) and assess its biocompatibility. Therefore, nine progesterone formulations were prepared and characterized. Data analysis revealed only one formula of P showed nanosized particle (1-100 nm) with an average particle size (95±5 nm), and spherical shape as seen by Transmission Electron Microscope(TEM). Motile spermatozoa were separated from frozen-thawed semen by a swim-up procedure and capacitated in IVF-TALP medium with NP or P or without treatments (control) and incubated for 3h at 38 C and evaluated every 1 hour (h) interval. Ovarian oocytes were matured and fertilized *in vitro* with frozen-thawed bull sperm capacitated in vitro with NP or P or control (without NP, P) and incubated at 39C in 5% CO2 incubator for 24h and then examined for evidence of fertilization. In conclusion, this study demonstrates that nanosized progesterone is highly efficient for sperm capacitation. In addition to the use of nanosized progesterone in sperm capacitation produces more fertilized oocytes than the progesterone after *In Vitro* Fertilization (IVF).

Keywords. Capacitation; *In vitro* fertilization; Nanoparticles; Progesterone; Spermatozoa.

Introduction

Nanoparticle is defined as a particle with dimensions ranged from (1 to100) nano-meters, [1]. The properties of nanosized particles differ from larger samples of the same material such as ultra-small size, large surface area to mass ratio and high reactivity. These properties improve the functions of the traditional material [2]. In addition, the ultra-small size of nanoparticles facilitate the penetration of particles through the small capillaries of tissues and cells and have main effect on the physiology of such cells [3]. also nanoparticles can transport across cell membranes, into the mitochondria [4].

Application of nanoparticles leads to considerable potential biological effects in animal and human cells [5]. On the other hand nanoparticles may have toxicological effects in certain concentrations [6,7].

There are different factors affecting the capacitation of sperm *in vitro* such as capacitating medium, and incubation periods. Consequently, these factors would affect the motility, viability and acrosome reaction rates. Mammalian sperm can undergo the acrosome reaction after complete capacitation in response to a number of physiological and pharmacological stimuli such as progesterone (P). P has been reported to affect several sperm functions especially capacitation and acrosome reaction according to Thérien et al.[8]. The effects of progesterone on spermatozoa are mediated via progesterone binding sites/progesterone receptors (PRs) on the acrosomal membrane. Different types of PRs have been shown to be present on the sperm plasma membrane. These are plasma membrane Ca2+ channel (PR1), a membrane-associated protein tyrosine kinase (PTK; PR2), and a plasma membrane chloride channel (PR3) [9]. Progesterone stimulates Ca2+ influx in the spermatozoa through PR1. Also, progesterone stimulates tyrosine phosphorylation of sperm proteins causing hyper activation [10] with an increase in cAMP levels [11]. The tyrosine kinase-associated PR (PR2) is responsible for the effect of progesterone on the hyperactive motility and acrosome reaction.

The main problem of natural progesterone is its low aqueous solubility. Because it is hydrophobic drug so it needs size reduction and surface modifications through preparation of nanoparticles.

In Vitro studies is a technique used to solve many problems associated with *in vivo* [12, 13].

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In this study we use bovine spermatozoa to evaluate the effect of nanoparticles on sperm capacitation. Sperm capacitation is a biochemical process characterized by membrane changes like cholesterol efflux, increase the membrane fluidity and ends by hyperactivation (HA) and acrosome reaction (AR) of spermatozoa [14].
In this study we investigate the biological effect of nanoparticles on sperm *in vitro* without *in vivo* exposure of animal to nanoparticles.

Material and Methods

All reagents, Chemicals and Media were from Sigma-Aldrich (Egypt). Stearic acid was provided by El Gomhoria CO. (Egypt).

Preparation of nanosized progesterone

Solvent precipitation method was used for preparation of the progesterone nanoparticles with some modifications [15].

Nine progesterone formulations were prepared by mixing of different concentrations of progesterone (P) (2, 10, 20 mg) with different amounts of stearic acid (SA) powder in different ratios (5, 10, 20%) in ethanol (1mL) (W/W) as seen in Table 1. The best formula was selected for sperm capacitation.

Table 1. Formulations of Progesterone nanoparticles.

Formula(NO)	Progesterone : Stearic acid ratio
F1	2 mg P : 5% SA
F2	10 mg P : 5% SA
F3	20 mg P : 5% SA
F4	2 mg P : 10% SA
F5	10 mg P : 10% SA
F6	20 mg P : 10% SA
F7	2 mg P : 20% SA
F8	10 mg P : 20% SA
F9	20 mg P : 20% SA

Charaterization of nanosized progesterone nanoparticles

The morphology of the progesterone nanoparticles were characterized by Transmission Electron Microscope (TEM) JEM-2100 (JEOL- td, Tokyo, Japan)
Particle size analysis
The PCS4700 system (Malvern Instruments, Malvern, UK) was used to determine the z-average particle sizes.
Zeta potential analysis
Zeta potential of the nanosuspension samples was determined using the Zeta Sizer 2000 (Malvern Instruments).

Yield of nanoparticles
The progesterone nanoparticles were separated by centrifugation and then freeze- dried using Freeze – Dryer, FDu-7003, (Kyeonggi-do, Korea) to yield the product.
The product yield of nanoparticles was calculated as follows:

$$\text{Nanoparticles yield} = \frac{\text{Weight of Nanoparticles}}{\text{Weight of initial Particles}} \times 100$$

Effect of nanosized progesterone on sperm capacitation

Separation of motile spermatozoa
Motile spermatozoa were separated according to the procedure of Abd-Allah [16] with some modifications.
Treatment of bovine spermatozoa with nanosized progesterone
According to the results of experiment 1, the nanosized progesterone below 100 nm of dimension was chosen for sperm capacitation. The Sperm suspension was divided into 3 equal sizes in 3 test tubes and submitted to different treatments: Control (IVF-TALP medium) P (IVF-TALP + P (10µg/mL)) and NP (IVF-TALP + NP(10µg/mL). After that, all test tubes were incubated at 39ºC for 3h.

Assessment of Sperm Capacitation

All fractions were evaluated for the following parameters during varying incubating periods.
Assessment of the hyperactivation motility
Hyper-activation Motility (HAM) measurements were performed according to the method of Fujinokiet al., with some modifications and was observed at 1, 2 and 3 h of incubation [17].
Assessment of Sperm Viability
Trypan Blue exclusion method was used for assessment of bovine spermatozoa viability according to Kitiyanant, Y et al. [18].
Assessment of acrosome reaction.
The Coomassie Blue G staining was used for evaluation of acrosome reaction [19].

The *in vitro* fertilization ability of spermatozoa

The nanosized progesterone below 100 nm of dimension was chosen for *in vitro* fertilization.

Oocyte collection and culture
Ovaries were collected and prepared for maturation according to the procedure of Abd-Allah [16].
The sperm concentration was determined using a haemocytometer. Aliquots of IVF-TALP (5–9 µL, containing approximately 2 10^6 motile sperm) were then added to the droplets containing 10-12 matured oocytes. IVF was performed by dividing in vitro matured

oocytes randomLy into 3 different groups. The first group was kept cumulus-enclosed after in vitro maturation (IVM) and fertilized in IVF-TALP medium (group 1: Control). The second group was fertilized in IVF-TALP medium supplemented with sperm capacitated with 10ug/mL progesterone (group 2: IVF-TALP+P),and the third group fertilized in was IVF-TALP medium supplemented with sperm capacitated with 10ug/mL progesterone Nanoparticles (group 3: IVF-TALP+NP), respectively. The experiment was replicated ten times.

Statistical analysis

Data are represented as the mean ± S.D. Significant differences among the groups were analyzed using one-way ANOVA test and post hoc comparisons using Tukey's test (SPSS18 software; SPSS, Inc., Chicago, IL, USA). Differences were considered significant at $P<0.05$.

Results

Characterizations of different formulations of progesterone

Detailed characterizations of different formulations of progesterone are briefly shown in Table 2.

Table 2. Characterization of different formulations of progesterone.

Formula (NO)	Structure of Formula	particle size(nm)±SD	zeta potential (mv)±SD	Yield (%)
F1	2 mg P: 5% SA	800±150	-25±9	35%
F2	10 mg P: 5% SA	470±32	-27±7	45%
F3	20 mg P: 5% SA	400±21	-32±5	60%
F4	2 mg P: 10% SA	260±120	-30±8	45%
F5	10 mg P: 10% SA	180±45	-36±6	60%
F6	20 mg P: 10% SA	165±15	-39±6	65%
F7	2 mg P: 20% SA	150±25	-43±7	70%
F8	10 mg P: 20% SA	95±10	-48±5	90%
F9	20 mg P: 20% SA	120±12	-49±6	75%

Data were expressed as Means ± SE, different alphabetical superscripts in the same rows (a, b, c, d) are significant at least at $P< 0.05$.

As seen in Table 2 only one formula of Progesterone (F8) showed an average particle size below 100 nm (95±5 nm) and used in this study. However other eight formulations were found that their particle sizes were above 100 nm so they were not included into the next experiments.

Comparing all progesterone formulations indicated that the F8 are better than other formulations in terms of yield percentages in addition, the average particle size decreased in F8 ($P> 0.05$).
The morphology of the progesterone nanoparticles was characterized by Transmission Electron Microscopy (TEM). As seen in Figure. 1 (A, B) nanoparticles are spherical and homogenous

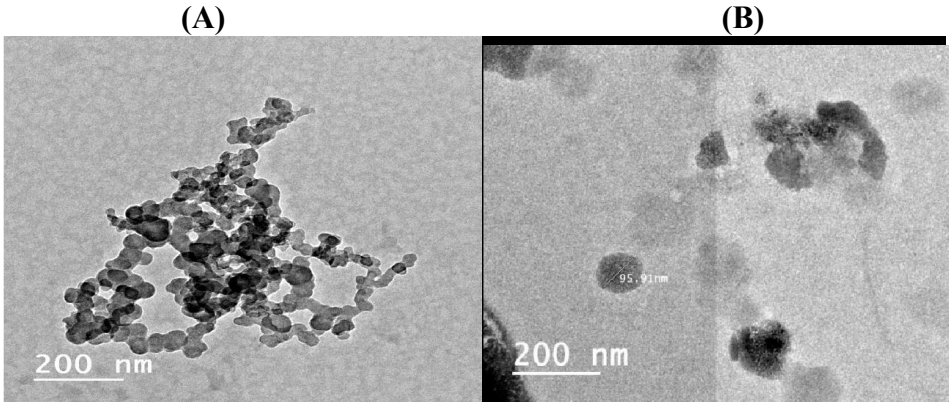


Figure. 1(A) TEM images of progesterone nanoparticles, **(B)** TEM images of progesterone nanoparticles (F8)
The statistical analysis showed effects of treatment ($P>0.05$) and incubation time ($P> 0.05$).

In vitro sperm capacitation

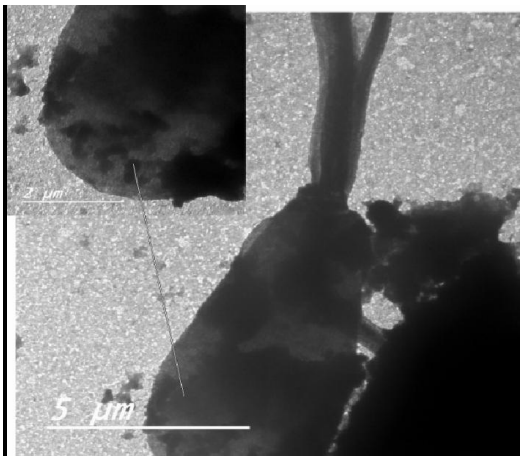


Figure. 2 TEM images of progesterone nanoparticles (F8) localized in side the sperm head

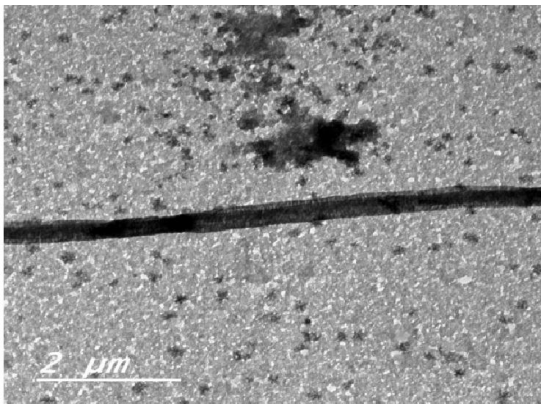
Treatment effect: HAM, Viability and AR were affected by treatment. A greater HAM and AR spermatozoa was observed for nanosized progesterone ($P < 0.05$) (Table 3).

Table 3. Effect of nanosized Progesterone on Hyper-activitated Motility, Viability and Acrosme Reaction of bull spermatozoa during sperm capacitation after 3 h incubation time.

Treatments	Sperm Characteristics		
	Hyperactivated Motility %	Viability %	Acrosome Reaction %
Control	15^{a} ±5	30^{a} ±2.5	20^{a} ±4.5
Progesterone	15^{a} ±3	35^{a} ±4	25^{a} ±2.5
Nanosized Progesterone	40^{b} ±2.5	70^{b} ±3.5	45^{b} ±3

^{a,b} Means values within a column with different superscripts differ significantly ($P<0.05$). n=10 for each treatment.

Time effect: HAM and AR were greatest at 2 h and viability at 1h as compared with other incubation times Tables 4, 5, 6. Also long incubation for 3h had bad effect on sperm viability and motility. As seen in Figure.2,3 localization of nanoparticles inside sperm head and tail respectively.



Figre. 3 TEM images of Progesterone nanoparticles (F8) localized in the tail membranes

Effect of nanosized progesterone on Sperm Hyperactivated Motility

Table 4 summarizes the analyzed data for this experiment.

Table 4: Effect of nanosized Progesterone and incubation time (hours) on hyperactivity of bovine spermatozoa.

Treatments	Incubation time (h)		
	1	2	3
Control	$25^{a\,A}$ ±2.5	$40^{b\,A}$ ±3	$15^{c\,A}$ ±3
Progesterone	30^{Bb} ±3.5	50^{bB} ±2.5	15^{bB} ±2.5
Nanosized Progesterone	50^{cC} ±4	70^{cC} ±1	40^{cC} ±2

^{a,b,c} Mean values within a row with different superscripts differ significantly ($P<0.05$). ^{A,B,C} Means values within a column with different superscripts differ significantly ($P<0.05$). n=10 for each treatment at specific time.

The changes in the motility rate of sperm were evaluated by analyzing the change in movement pattern after incubation for 1 and 3 h in different formulations of progesterone nanoparticles as shown in Table 4, the percentages of HAM were highly significant in nanosized progesterone compared to the control and progesterone treatments.

Effect of nanosized progesterone on Sperm Viability

The results obtained for viability presented a significant difference (P<0.05) in the percentage of live sperm after 3h of incubation in the treatment that received NP as seen in Table 5.

Table 5. Effect of different formulations of Progesterone Nanoparticles and incubation time (hours) on viability of bovine spermatozoa.

Treatments	Incubation time (h)		
	1	2	3
Control	55^{Aa} ±2	45^{aA} ±2.5	30^{aA} ±3
Progesterone	60^{Bb} ±1.5	50^{bB} ±3	45^{bB} ±2.5
Nanosized Progesterone	85^{cC} ±2.5	80^{cC} ±2	70^{cC} ±3.5

^{a,b,c,} Mean values within a row with different superscripts differ significantly (P<0.05).^{A,B,C} Means values within a column with different superscripts differ significantly (P<0.05). n=10 for each treatment at specific time.

Effect of nanosized Progesterone on Sperm Acrosome reaction

As seen in Table 6. AR significantly increased in PN at 2h incubation as compared to progesterone and control (75 vs. 50, 40%, respectively,

Table 6. Effect of nanosized progesterone and incubation time on acrosome reaction of bovine spermatozoa.

Treatments	Incubation time (h)		
	1	2	3
Control	30^{aA} ±2.5	40^{aA} ±2	20^{aA} ±1
Progesterone	35^{bB} ±3	50^{bB} ±2.5	25^{bB} ±2.5
Nanosized Progesterone	55^{cC} ±5	75^{cC} ±5	45^{cC} ±4

^{a,b,c,d,e} Mean values within a row with different superscripts differ significantly (P<0.05). ^{A,B,C} Means values within a column with different superscripts differ significantly (P<0.05). n=10 for each treatment at specific time.

In vitro fertilization

A summary of the results of in vitro fertilization is shown in Table 7. The fertilization rate was highest at nanosized progesterone (55%) than for control and progesterone (35% and 45% respectively). Figure. 4.

Table 7. Fertilization rate according to the results of hyperactivated motility (HAM) and acrosome reaction (AR) of nanozsized progesterone.

Treatments	Inseminated Oocytes	Mean of fertilized Oocytes±SD	Fertilization Rate (%)
Control	100	35± 3	35^{a}
Progesterone	100	45±2.5	45^{b}
Nanosized Progesterone	100	55±4.5	55^{c}

^{a,b} Means values within a column with different superscripts differ significantly (P<0.05).n=10 for each treatment.

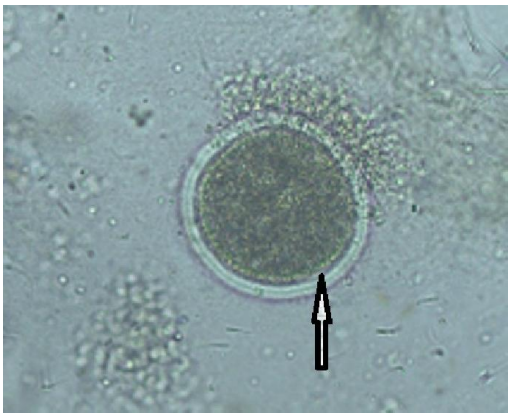


Figure. 4: A representative fertilized ocytes after 22 h fertilization.

Discussion

According to our knowledge this is the first report of *in vitro* capacitation of bovine spermatozoa by nanosized progesterone. Comparing nine progesterone formulations, data analysis revealed that only one formula of Progesterone (F8) showed an average particle size below 100 nm (95±5nm) and this agreed with the standard dimensions which was reported by many organizations such as ISO, ASTM, NIOSH, SCCP, BSI and BAuA [1]. The prepared nanoparticles have an average particle Size ranged from 95 to 480 nm according to the amount of stearic acid. Our results showed that the amount of stearic acid had main effect on the particle size, zeta potential, and the yield %. By increasing the amount of stearic acid to progesterone, the average particle size decreased significantly, and zeta potential of nanoparticles increased, also the yield % increased which may be due to the repulsion occurring between the nanoparticle surfaces and the walls of the glass vessels upon transfer. It was presumed that the ratio between progesterone and stearic acid remain the same after nanoparticles preparation.

For induction of the acrosome reaction, the culture media for sperm capacitation was supplemented with different agents, for example in cattle the capacitation media supplemented with progesterone, which stimulates capacitation of bovine spermatozoa [8, 20-22]. Also Parrish described the behavior of spermatozoa during capacitation and the first changes in acrosome morphology appear after 2 h [23].

Several studies evaluate the biological and toxicological effects of nanoparticles on sperm and oocytes. The nanoparticles may have positive effect or toxicological effect on sperm according to Taylor, U et al. and Asharani, P., et al.[24-28].

In our study we used progesterone as nanosized which differ from normal progesterone in their chemical and physical properties such as ultra-small size, large surface area to mass ratio and high reactivity so the (NP) are small enough to penetrate through sperm plasma membrane, so can easily affect the physiology of sperm cells by increasing the intracellular calcium. Also progesterone stimulates tyrosine phosphorylation of sperm proteins causing hyperactivation with an increase in cAMP levels. The tyrosine kinase-associated PR (PR2) is responsible for the effect of progesterone on the hyperactive motility and acrosome reaction.

We observed that nanosized progesterone (NP) is highly efficient for sperm capacitation and also improved the functions of sperm. Our results showed that with prolongation of time for 3 h, sperm treated with NP lost its hyper activated motility and viability and the others have the same results [8, 29].

Conclusions

Collectively, based on the finding of the present study, it can be concluded that bovine spermatozoa incubated with nanosized progesterone for 2h revealed increased sperm hyper-motility and acrosome reaction as compared to progesterone or control as well as it produced the highest percentage of fertilized oocytes. The fertilization rate was highest at nanosized progesterone (55%) than for control and progesterone (35% and 45% respectively), this is may be due to the improvement of sperm functions with nanosized progesterone. So this study is considered a promise for dissolving many problems associated with IVF due to bad quality of sperm

Further studies will be necessary to elucidate the specific biochemical and molecular changes which result from the action of nanosized progesterone in the capacitation process of bovine sperm.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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