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Comparative effect of saline solutions as diluents on *in vitro* semen storage, egg fertility and hatchability in Turkey hens

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Target audience: *Turkey breeders, Poultry farmers, Researchers, Animal scientists*

Abstract

The high viscosity and concentration of turkey semen necessitates its dilution prior to artificial insemination. The comparative effects of Modified Ringer's Solution (MRS), Normal Saline (NS) and Dextrose Saline (DS) as diluents on semen quality and fertility in turkeys were investigated. Semen pooled from 20 toms was divided into four: Undiluted Semen (US), others were diluted at 1:1 with MRS, NS or DS and stored at ambient temperature (27.75±0.25℃). Progressive Spermatozoa Motility (PSM) was measured hourly till it was below 50%. Sixty turkey hens were inseminated with the treatments for two successive days, and fertility assessed over ten weeks. At first hour of storage, PSM was greater than 90% in all treatments while it was significantly (P<0.05) higher in MRS (58.3±2.9%) and DS (56.7±1.7) than US (43.2±2.9%) and NS (41.7±1.7%) at the fourth hour. Fertility at the first six weeks post insemination was similar among the treatments. Values at the first 3 weeks were 81% to 97%. At week 8, it was significantly (P<0.05) higher in NS (20.83%) than others (0%). Modified Ringer's solution, normal saline and dextrose saline are suitable as semen diluents for insemination in turkeys. However, modified Ringer's solution and dextrose saline stored semen better in vitro.

Keywords: *Dextrose saline; Normal saline; Ringer's solution; Saline solutions; Turkey fertility; Turkey semen*

Description of Problem

Turkey semen is relatively high in viscosity and concentration when compared to other poultry species (1). Its dilution prior to Artificial Insemination (AI) is therefore very necessary, to increase the volume of semen and serve a wider coverage of hens to be inseminated. This dilution is done using semen diluents or extenders, which are aqueous solutions also utilised for long or short term storage of semen. Semen storage is necessary in AI practice to maintain the viability of sperm cells *in vitro* prior to insemination. Dilution of semen is expected to improve the reproductive efficiency of the males thereby reducing the costs associated with AI.

The development of semen diluents began with the use of sodium chloride solutions (2). More complex diluents containing osmotic regulators (NaCl, KCl), energy sources (glucose), buffers (Bicarbonate, Tris) and antibiotics are now being developed. A saline solution is a mixture of sodium chloride in water and may have other salts in addition too. The common types of saline solutions associated with cell biology are normal saline (NS), Ringer's solution, dextrose saline, phosphate buffered saline, TRIS – buffered saline, Hank's balanced salt solution (HBSS), etc. A modification of Ringers solution has

been reported as a semen diluent (3) and was used as a diluent for chicken semen and insemination (4). Normal saline and dextrose saline are sterile solutions used for fluid and electrolyte replenishment among other uses.

Evidence from many studies indicates that there is no standard diluent for poultry semen due to the variations (such as number of sperm cells inseminated, semen storage time, insemination frequency and method of fertility assessment) associated with these experiments (5). These among other factors differentiate the benefits of the various diluents depending on the goal of the farmer or researcher.

For the adoption of AI to be economically viable to the local farmers, semen dilution is important and should be done under conditions practical to farmers for easy adoption. Such practical conditions may involve insemination immediately after semen collection and processing and as such, will exclude the need to store semen in a refrigerated condition as most local farmers may lack access to a constant electricity supply. The availability and cost of semen extenders are other factors that may limit its use.

The objective of this study was therefore to compare the efficacy of normal saline and dextrose saline as diluents, with modified Ringer's solution and undiluted semen, on the quality of semen stored *in vitro* at room temperature and on egg fertility in inseminated turkey hens. This is to avail the local farmers the use of available and affordable sterile solutions as diluents in turkey insemination without compromise on fertility or hatchability of the eggs.

Materials and Methods

The research was carried out at the poultry unit of the Teaching and Research Farm and the Animal Physiology Laboratory, Department of Animal Science, of the University of Ibadan, Ibadan, Nigeria.

Semen collection and dilution

Semen was harvested from twenty locally-adapted toms (16 months old) using the abdominal massage method (6), pooled and divided into 4 portions. Three portions were each immediately diluted at a ratio of 1:1 with modified Ringers solution, 0.9% normal saline or 1% dextrose saline (with all three diluents warmed to 37°C prior to dilution) while the fourth portion was undiluted. Each portion was replicated into three eppendorf tubes. The tubes containing the samples were completely wrapped with dry cotton wool and stored at ambient temperature (27.75±0.25°C). The composition of each of the diluents is shown in Table 1. Normal saline (0.9% sodium chloride) and dextrose saline (5% dextrose saline) were purchased from a pharmaceutical shop. The osmolarity of 5% dextrose saline is 560m Osm/L. This value is much higher than the osmolarity range of 250 to 460 mOsm/L recommended (5) for preserving the fertilising ability of sperm cells. It was therefore reconstituted by diluting one part of the 5% dextrose saline with four parts of normal saline to give 1% dextrose in 0.9% saline solution prior to use. The osmolarity of the reconstituted dextrose saline was 363 mOsm/L. Osmolarity (mOsm/L) was calculated as:

Number of ions in solute x 1000 x Weight of solute (g/L)/ Molecular weight of solute

In vitro semen evaluation

In vitro semen evaluation was done on the samples during storage at ambient temperature (27.75±0.25°C). They were analysed hourly, for progressive spermatozoa motility, plasma membrane integrity and spermatozoa liveability till the fourth hour after semen collection when sperm motility had dropped below 50% for some samples.

Table 1: Composition of diluents

Constituent (g/100ml distilled water)	ModifiedRinger's Solution	Normal saline	Dextrose Saline
Sodium chloride	0.68	0.90	0.90
Potassium chloride	0.17	-	-
Calcium chloride	0.06	-	-
Magnesium sulphate	0.03	-	-
Sodium bicarbonate	0.24	-	-
Dextrose	-	-	1.00
Osmolarity (mOsm/L)	333	308	363
pH	6.74	6.79	6.70

Table 2: Experimental layout showing diluents, volume of semen and estimated motile sperm cells inseminated

Treatment groups (diluents)	Ratio of semen to diluent	Semen volume inseminated (mL)	Estimated motile sperm cells inseminated ($x10^6$)
Undiluted semen ($US_{0.02}$)	1:0	0.02	107.3
Undiluted semen ($US_{0.01}$)	1:0	0.01	53.7
Modified Ringer's solution	1:1	0.02	53.7
Normal saline	1:1	0.02	53.7
Dextrose saline	1:1	0.02	53.7

Progressive spermatozoa motility: A drop of raw semen was placed on a sterile glass slide and 1 to 2 drops of warmed (37℃) sodium citrate solution was immediately added. A cover slip was placed over it and it was examined at a magnification of x400. Percentage motile sperm cells were determined by subjective scoring between 0 and 100% (7).

Plasma membrane integrity: This was determined using the hypo- osmotic swelling test (8). A hypo-osmotic solution was prepared by dissolving 1g of sodium citrate in 100mL distilled water to give a solution with an osmolarity of 100mOsm/L. Semen sample of 0.1mL was then mixed with 1mL of hypo-osmotic solution (1:10 dilution). The solution was placed in a warm bath at 37 ℃ for 30 minutes. The sperm cells were thereafter viewed under a light microscope at a magnification of x400. Swelling of the cells as characterised by curving of the tail was noticed. The number of cells with curved tail was estimated and expressed as a percentage of

total cells under view to determine the plasma membrane integrity.

Spermatozoa liveability: The percentage live cell was determined by staining semen sample with Eosin-Nigrosin stain. Due to the high sperm concentration of turkey semen, the semen sample to be stained was first diluted with warmed (37°C) sodium citrate at a ratio of 1 to 20 (semen to sodium citrate) to make counting of cells possible after staining. Thereafter, a drop of diluted semen sample was placed on a glass slide and then a drop of Eosin-Nigrosin stain was added. It was gently mixed and smeared on another clean glass slide with the slide used to mix. Excesses were blotted off and the stained slide was allowed a few seconds to air-dry. It was then viewed under the microscope at a magnification of x400. The cells which absorbed the stain were regarded as the dead cells. The live cells were determined by subtracting the dead cells from the total cells under view. Liveability was calculated by expressing the live cells as a percentage of the total cells viewed (7).

Semen collection and insemination

Sixty locally-adapted turkey hens, 14 months old,were randomly divided into 5 treatment groups of3 replicates with 4 hens each, in a completely randomised design. Semen was collected from the toms, pooled and divided into 4 portions. Three portions were diluted at a ratio of 1 to 1 with normal saline, dextrose saline or modified Ringer's solution with compositions in Table 1 while a portion was left undiluted as done in the *in vitro* assessment. Prior to dilution, the diluents were warmed to a temperature of 37°C. The experimental layout indicating the respective treatments and the volume inseminated is presented in Table 2. The oviduct of each hen was everted and semen was deposited into it at a depth of about 2.5cm. The period of semen collection, dilution and insemination of all the hens was within 45 minutes and it was done after 5pm. Insemination was done for 2 successive days only after which eggs were collected. A sample of pooled semen inseminated was analysed for sperm concentration and sperm motility (7) to estimate the number of motile sperm cells inseminated.

Fertility Assessment

The day after the second insemination marked the day for the first egg collection. Eggs were collected daily from each treatment group, marked and stored on paper trays with broad end up at a temperature range of 24 °C to 26 °C and relative humidity of 70% – 85% for a week. They were incubated on weekly basis, to determine fertility and hatchability. Egg collection and weekly incubation were done for a period 10 weeks. At day 25 of incubation, candling was done and all candling clears were removed in addition to all unhatched eggs at day 28 for break out analysis. Infertile eggs were separated from those with embryonic deaths. The candling clears were broken-out and the numbers of infertile eggs were recorded. Infertile eggs were those eggs which upon break-out, were devoid of any form of embryonic mass. Eggs that had a form of embryonic mass upon break-out were categorised as fertile eggs with embryo mortality. All hatched poults and dead embryo were counted and regarded as the number of fertile eggs. Percentage fertility was calculated by expressing the total fertile eggs as a percentage of all eggs set while hatchability was all hatched poults expressed as a percentage of all fertile eggs.

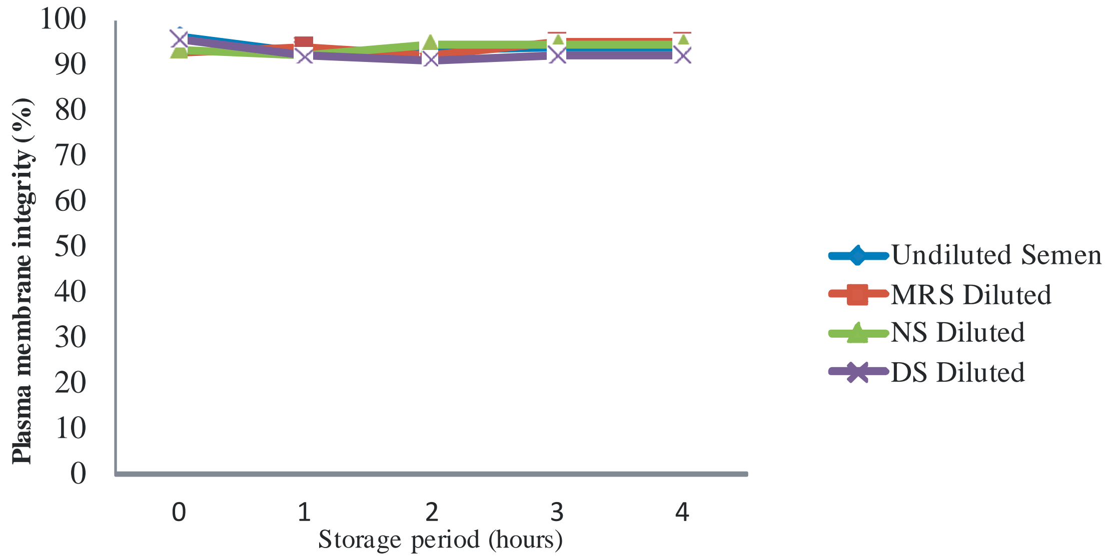

Figure 1: Plasma membrane integrity of undiluted semen and semen diluted with modified Ringer's solution (MRS), normal saline (NS) or dextrose saline (DS) and stored for four hours at room temperature (27.5 ℃ - 28.0℃). There was no significant difference (P>0.05) among the values at each of the storage time. Values are depicted as mean of samples.

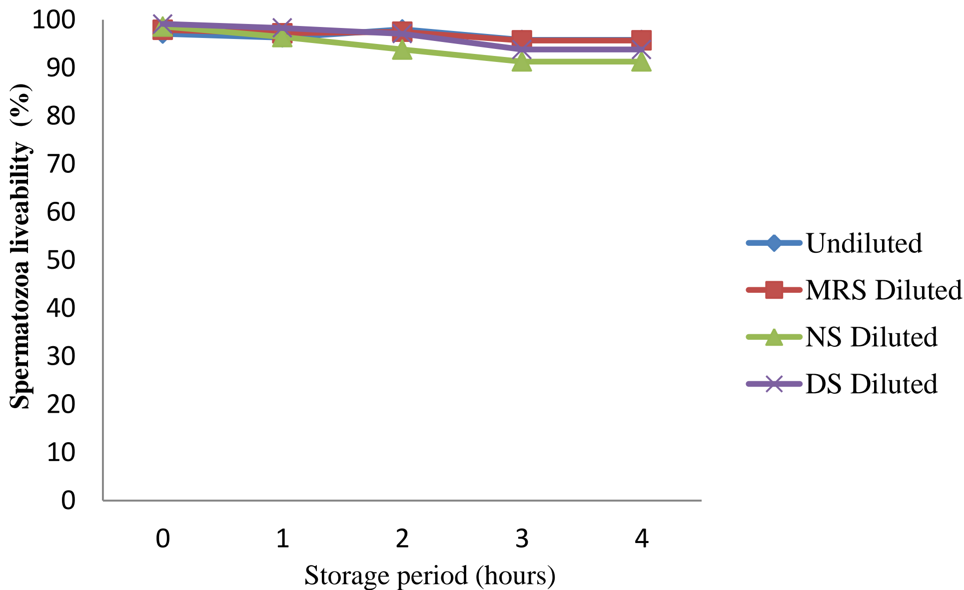

Figure 2: Spermatozoa liveability of undiluted semen and semen diluted with modified Ringer's solution (MRS), normal saline (NS) or dextrose saline (DS) and stored for four hours at room temperature (27.5 ℃ - 28.0℃). There was no significant difference (P>0.05) among the values at each of the storage time. Values are depicted as mean of samples

Statistical analysis

Data collected and recorded in percentage were subjected to square root transformation. Analysis of variance was done on square root transformed data using general linear model of SAS (9) and means were separated using Duncan's multiple range test of same software.

Results

The Progressive Spermatozoa Motility (PSM) of Undiluted Semen (US) and semen diluted with Modified Ringers Solution (MRS), Normal Saline (NS) and Dextrose Saline (DS) over 4 hours at room temperature (27.75±0.25℃)is presented in Table 3. At the zero and first hour, PSM was not significantly (*P*>0.05) different among treatment groups. It ranged from 91.7% to 95.0%, but by the 4th hour, it was significantly (*P*<0.05) lower in NS (41.7%) than the other groups.

The result on Plasma Membrane Integrity (PMI) and spermatozoa liveability are presented in Figures 1 and 2, respectively. There was no significant (*P*>0.05) difference among parameters at each time of assessment. The PMI and liveability across all the groups and the time were above 90%.

The result on fertility assessment is presented on Table 4. Fertility declined with time post insemination. There was no significant difference (*P*>0.05) among the treatment groups at each of the weeks up till the 6th week post insemination with values of above 76% at the first four weeks. Fertility at the 7th week post insemination, in NS (34.3±5.6%) was significantly (*P*<0.05) higher than all other groups except DS (13.9±7.4%) and by the 8th week, was significantly (*P*<0.05) higher than all treatment groups which were already 0%. Observations at the 9th week indicated 0% fertility across all treatment groups. This is an indication that despite similarities in fertility among the groups at the first six weeks post insemination, NS group had a relatively longer duration of fertile period compared to the other groups.

The hatchability of Eggs from the turkey hens is presented in Table 5. Results were presented only for the first 5 weeks post insemination because of the drop in the number of fertile eggs thereafter. Hatchability is expressed as a percentage of fertile eggs hence the error margin is expected to be very large if weeks after the 5th is analysed. There was no significant difference in hatchability among all the treatment groups at each of the weeks. Values ranged from 78.8±5.7% to 100±0.0 across the treatment groups and the weeks post insemination.

Discussion

The undiluted semen depended solely on the contents of the seminal plasma which supported motility that was comparable to semen diluted with Dextrose Saline (DS) and Modified Ringer's Solution (MRS) up till 2 hours. The MRS, Normal Saline (NS) and DS utilised as diluents in this study have osmolarity and pH that fall within the range of 250 – 460 mOsm/L and 6 – 8, respectively (Table 1) that is required for diluents to maintain optimal fertility of the sperm cells (5). It has been reported, that osmotic balance is the primary requirement to maintain turkey sperm cells *in vitro* and semen held up to an hour with diluents osmotically balanced for turkey sperm cells will produce optimal fertility (10).Thus, results indicating maximum sperm motility among the treatments without significant difference at 1 hour in this study corroborated this report. The result of this study further suggests that progressive sperm motility of above 80% can be obtained at the 2nd hour of storage with the undiluted semen and that dilution with MRS and DS which may be an indication for optimal fertility too, if such semen is inseminated. Normal saline contains only NaCl which is expected to maintain osmotic balance. The absence of other constituents may have resulted in its

lowered sperm motility at the 2nd hour when compared to the other diluents. Dextrose saline contained an energy source in addition to NaCl which is dextrose and this may probably be responsible for sustenance of the sperm cells, hence a relatively higher motility when compared to NS. However, MRS did not have an energy source but motility was sustained for as long as DS. This may have resulted from the reduced NaCl in the MRS when compared to DS and NS thus leading to a reduction in ATP required in maintaining membrane stability. Hence, more energy was available for sperm motility. The reduction in NaCl in modified Ringer's fluid has been reported to affect a cut in energy spent on sodium pump mechanism in perfused hearts (11). This will make more energy in form of ATP available for other cell processes. The additional salts like potassium chloride present in MRS may have made up for the shortfall in NaCl in maintaining membrane stability as potassium and chloride ions are also important components of the intracellular and extracellular fluid. They also play important role in maintaining the resting state of the cell (12). The presence of calcium in the form of calcium chloride in MRS may have improved motility as calcium was known to increase sperm motility *in vitro* (13).

The plasma membrane integrity and liveability of the cells maintained at maximum for up to four hours is an indication that despite the drop in motility of the cells at this time, they were still alive and able to maintain their structural and functional membrane integrity. This supports reports that the percentage of dead spermatozoa was at the least and constant for up to four hours after semen collection but increased significantly after 24 hours at a room temperature of 22℃ (14).

Table 3: Progressive spermatozoa motility (%) of undiluted semen and semen diluted with modified Ringer's solution, normal saline or dextrose saline during storage at ambient temperature (27.5 ℃ - 28.0℃). Values are shown as mean ± standard error.

Treatments (diluents)	Storage period				
	0 hour	1 hour	2 hours	3 hours	4 hours
Undiluted semen	93.3±1.7	95±0.0	93.3±1.7^{a}	66.7±3.3^{b}	48.3±2.9^{b}
Modified RS	93.3±1.7	91.7±1.7	90.0±0.0^{a}	83.3±1.7^{a}	58.3±2.9^{a}
Normal saline	93.3±1.7	91.7±1.7	78.3±1.7^{b}	63.3±1.7^{b}	41.7±1.7^{c}
Dextrose saline	91.7±1.7	91.7±1.7	86.7±3.3^{a}	83.3±1.7^{a}	56.7±1.7^{a}

a, b, c - means along same column with different superscripts are significantly (P<0.05) different; Mean± standard error; RS – Ringer's Solution

Table 4: Egg fertility (%) from turkey hens inseminated with undiluted semen and semen diluted at 1:1 with modified Ringer's solution, normal saline or dextrose saline. Values are shown as mean ± standard error.

Weeks (post insemination)	Treatments (Semen volume inseminated)				
	Undiluted semen (0.02mL)	Undiluted semen (0.01mL)	Modified RS (0.02mL)	Normal saline (0.02mL)	Dextrose saline (0.02mL)
1	81.5 ±11.3	81.0 ±6.3	91.7 ±3.2	84.4 ±6.2	87.1 ±5.7
2	97.0 ±3.0	81.0 ±1.7	84.7 ±1.4	81.0 ±1.7	90.3 ±5.0
3	88.9 ±7.4	85.1 ±11.5	94.4 ±5.6	81.5 ±7.4	85.9 ±0.8
4	95.9 ±34.0	84.7 ±9.7	79.6 ±15.2	81.8 ±9.8	76.7 ±2.7
5	67.5 ±14.2	72.2 ±14.7	58.3 ±22.1	61.7 ±7.3	61.1 ±5.6
6	29.2 ±15.0	24.6 ±12.3	26.1 ±3.9	50.0 ±14.4	59.2 ±20.4
7	$4.2±4.2^{bc}$	$0.0±0.0^{c}$	$0.0±0.0^{c}$	$34.3±5.6^{a}$	$13.9±7.4^{ab}$
8	$0.0±0.0^{b}$	$0.0±0.0^{b}$	$0.0±0.0^{b}$	$20.8±4.2^{a}$	$0.0±0.0^{b}$
9	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

a,b,c – means across same row with different superscripts are significantly ($P < 0.05$) different; RS – Ringer's solution

Table 5: Egg Hatchability (%) from turkey hens inseminated with undiluted semen and semen diluted at 1:1 with modified Ringer's solution, normal saline or dextrose saline Values are shown as mean ± standard error.

Weeks post insemination	Treatments (Semen volume inseminated)					
	Undiluted (0.02mL)	Undiluted (0.01mL)	MRS (0.02mL)	NS (0.02mL)	DS (0.02mL)	P-Value
1	86.7±6.7	91.9±4.2	78.8±5.7	94.9±5.1	96.3±3.7	0.1793
2	93.3±6.7	91.5±4.3	100±0.0	97.6±2.4	87.5±6.7	0.4226
3	87.3±2.2	97.4±2.6	93.3±6.7	91.7±8.3	86.2±7.9	0.6932
4	79.2±15.0	100±0.0	95.8±4.2	100±0.0	84.2±8.2	0.2785
5	89.7±5.2	94.4±5.6	91.7±8.3	82.2±9.7	83.3±16.7	0.8686

$P>0.05$ - Not significant; MRS –Modified Ringer's solution; NS – Normal saline; DS – Dextrose saline

The similarity in fertility of the groups inseminated with diluted semen and that inseminated with 0.02mL undiluted semen is an indication that the dilution effect of reduction in number of sperm cells had no negative effect on fertility. Thus, stressing the need for semen dilution to maximise its use. Despite the differences observed in sperm motility *in vitro* among undiluted semen and that diluted with modified Ringers solution, normal saline and dextrose saline, there was no significant difference in the fertility response to the diluents for up to 6 weeks post insemination. This implies that all the diluents can be suitably used to dilute semen prior to insemination to give optimal fertility. Results

from this study indicate a longer duration of fertile period for the normal saline diluted semen group, despite relatively lowered sperm motility during *in vitro* storage. This is also an indication that the mechanism for sperm storage *in vitro* is quite different from *in vivo*. Components like energy sources, buffers, antibiotics, osmotic regulators etc are required in the diluents to preserve the sperm cells from detrimental effects outside the animal's body. The moment the viable cells enter the oviduct, factors associated with sperm selection and storage in the sperm storage tubules is what they are exposed to. These factors are partly associated with the modulation of zinc which is abundant in the mucosa surrounding the SST (15) and calcium, which is abundant in the uterus (16). Zinc has been shown to reversibly suppress oxygen consumption by cells and acts as a membrane stabilizer (17), while calcium is known to stimulate both motility and respiration of chicken sperm cells *in vitro* (13). Hypothetically, sperm activation will therefore occur when sperm cells are released from the SST to the calcium rich uterine environment (16). The significantly higher fertility for the NS at week 7 could be an indication that they had better oviductal sperm storage. It was also an indication of their relatively longer duration of fertile period when compared to other treatment groups. The reason for this is yet not clear since the other diluents also contained the only salt i.e. NaCl present in it.

The hatch of fertile eggs were not affected by the diluents and values reported for the first 5 weeks after insemination were higher than 70.0% – 75.5% reported in literature (18). This implies that none of the diluents had any detrimental effect on embryo mortality as it is the primary component of hatchability.

Conclusions and Applications

1. The fertility response of normal saline and dextrose saline as turkey semen diluents compared well with undiluted and modified Ringer's solution diluted semen.
2. Modified Ringer's solution and dextrose saline showed better response to sperm motility during *in vitro* storage while normal saline had better fertility response from *in vivo* sperm storage.
3. The relatively lower sperm motility of semen diluted with normal saline during *in vitro* storage, and then a better fertility response during storage *in vivo* further suggests that the mechanism of sperm storage *in vitro* and *in vivo* are quite different.
4. Modified Ringer's solution, normal saline and dextrose saline are suitable as turkey semen diluents for insemination.

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