THE EFFECT OF pH ON THE LIVABILITY OF BULL SPERMATOZOA

by

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ABSTRACT

The effect of hydrogen-ion concentration on the livability of bull spermatozoa stored at 5-10° C was studied. Two well known and commonly used diluents were tried, namely, Egg yolk-citrate developped by Salisbury et al. in 1941 and Egg yolk-phosphate developped by Phillips and Lardy in 1939. Five % egg yolk was used in both cases. Split ejaculate technique was employed, whereby the semen from the same ejaculate was divided into 6 portions and subjected to 6 different pH treatments covering a range of 6.2 - 7.3 and 5.9 - 7.1 for citrate and phosphate buffers respectively. Percent live spermatozoa was calculated by direct microscopic counts on the first, second, and fifth day of storage. Data from 10 trials with citrate and 5 trials with phosphate were cellected and studied.

The results show that pH has a statistically significant effect on the livability of bull spermatozoa in both buffers. However no well defined optimum could be found, but rather a range. This being 6.6 - 6.8 for the citrate and 6.5 - 6.9 for the phosphate buffers. The differences in livability between ejaculates were statistically significant, poor ejaculates showing poor storage quality. The livability was always better in citrate than in phosphate buffer. Maximum livabilities in the pH range of 6.6 - 6.8 obtained in citrate was 76, 62, and 34% in the first, second and fifth day, respectively. Corresponding values for phosphate were 73, 52, and 8, in pH range of 6.5 - 6.9.

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INTRODUCTION

Historical

The history of artificial insemination is often dated back to 1322, when dubious reports assert that an Arab chief stole semen from an outstanding stallion belonging to an enemy chief and successfully inseminated his own mare. There was almost nothing known about semen until 1677, when Anton Van Leeuwenhock and his pupil, John Hamn, discovered spermatozoa in semen by the use of a magnifying lens identifying them as "animalcules" (Rice et al. 1957). The first reliable report on artificial insemination dates back to 1780 when Spalanzani, an Italian scientist, successfully inseminated female dogs (Rice et al. 1957). He also, in 1803, observed the effect of low temperature on the spermatozoa reporting that cooling was not detrimental to semen. Around 1900 Sand and Stribolt inseminated mares in Denmark, but the problem, however, did not receive serious attention until 1922 when a Russian, E.I. Ivanoff, started an extensive practice of artificial insemination in horses, sheep, and cattle (Rice et al. 1957). Following this work were the establishment of cooperative Artificial Breeding Associations by Edward Sorenson (1937) in Denmark and E. J. Perry (1938) in the United Stafes (Rottensten, 1964). Other countries followed in rapid succession. It is beyond the scope of this present work to assess the merits of artificial insemination. However, during the

past twenty-five years the practice has revolutionized the livestock industries and, in particular, the dairy industry all over the world. The following figures will more than emphasize the importance and rate of expansion of artificial insemination. The world total number of cows artificially inseminated in 1958 and 1960 were 22,486,101 and 48,392,000 respectively (E.J. Perry, 1960; W. Bielanski, 1963).

The Problem of Diluter

The expansion of artificial insemination has a respectable amount of research behind it. Research is carried out to improve the techniques of collecting, extending, storing and using the semen to attain success. One of the reasons for the success of artificial insemination, and also one of its primary merits, is the possibility of extending the semen in a suitable diluter so that a large number of cows can be inseminated by one ejaculate, instead of one cow as in natural matings. This has caused the research workers to direct their efforts toward finding a kind of medium in which spermatozoa could be stored for a reasonable length of time without losing their fertilizing ability. In order to prolong the survival time of the spermatozoa in vitro, particular research has been exerted toward suppressing the metabolic activity of the sperms, so that they will not exhaust their energy reserve, but will remain in a dormant state until insemination time. This objective has been met by various physical and chemical methods such as lowering the storage temperature or using chemical inactivators. Generally two preservation techniques are followed: (1) the preservation at above-freezing temperatures and (2) the preservation

at below-freezing temperatures. In the former case the metabolic activity of the spermatozoa is brought to a minimum but not to a complete cessation by cooling the semen to 4 - 5° C. In this state the time of preservation is rather short not extending more than a few days. In the latter case, however, the spermatozoa are cooled to -79° C or lower where their metabolism is almost completely stopped. It was found that spermatozoa retain sufficient livability to cause conception for years when kept at this low temperatures.

In either method of preservation the choice of diluter plays a decisive role in attaining a success. It is also highly important to appraise the physical, chemical and biological properties of a diluter which help in preserving the natural power of fertilization of the sperms. A successful diluter should have the following properties:

a. It should be isotonic with semen or blood.

b. It should possess a certain buffering capacity and a desirable pH.

c. It should contain proper amounts of minerals and metabolites essential to the sperms.

d. It should contain certain factors which prevent cold shock.

e. It should not contain any materials which might prove to be toxic to the sperms.

f. It is desirable that it should be cheap and easy to prepare and that it has a physical state that makes the sperms visible under the microscope.

There have been practically hundreds of various diluters proposed and numerous attempts have been made to improve them. Some have studied the effect of adding carbohydrates, enzymes, vitamins, glycerol and antibiotics. Others have tried to change some factors such as the amount of egg yolk, ratio between certain electrolytes and the level of acidity.

The objective of the present work was to investigate the effect of hydrogen-ion concentration on the livability of bull spermatozoa stored in egg yolk-phosphate and egg yolk-citrate buffers at refrigeration temperatures. The livability was measured by determining the percent motility and activity rating by direct microscopy after the two and five days of storage.

REVIEW OF LITERATURE

Diluters Used

Satisfactory and comparable results have been obtained with the following diluters when the semen has been used shortly after collection:

1. Egg yolk-phosphate (Phillips and Lardy, 1939). This diluter is composed of 0.2 gms. KH_2PO_4 and 2.0 gms. $Na_2HPO_4.12H_2O$ dissolved in 100 ml. of distilled water and mixed with an equal amount of fresh egg yolk.

2. Egg yolk-citrate (Salisbury et al.,1941). This contains 2.9 gms. $Na_3C_6H_5O_7.2H_2O$ in 100 ml. of distilled water mixed with an equal amount of fresh egg yolk.

3. Whole or skim milk (Thacker and Almquist, 1953). The milk is heated to 92°C for about 10 minutes and then cooled before use.

4. Illini Variable Temperature (IVT) diluter (VanDemark and Sharma, 1957). This diluter has the advantage over the others in that the sperms can be stored in it at room temperature for the same period of time as they can be stored in the above diluters at 5 - 10°C. It consists of 20.0 gms. sodium citrate dihydrate, 2.1 gms. $NaHCO_3$, 0.4 gms. KCl and 3.0 gms. glucose dissolved in 1000 ml. of distilled water. This solution is saturated with CO_2 by bubbling the gas into it for 10 minutes until the pH drops to 6.35. Ten percent fresh egg yolk is then incorporated in it.

The following are some diluters which have been less successful: Chick embryo, normal saline, various body fluids, glucose, egg yolk-

tomato juice, honey, muscle extract and others. The most commonly used diluents are the egg yolk-phosphate, the egg yolk-citrate, and the milk diluents.

The pH of Semen

One of the most important objectives of research workers in the field of artificial insemination has been to lengthen the storage life of semen. The studies along this line have primarily been directed towards acquiring sufficient knowledge concerning the metabolism of spermatozoa and the factors affecting it.

All living organisms have certain environmental requirements for their survival. Sperms are no exception. Hydrogen-ion concentration is a factor which affects many metabolic and enzymatic reactions in biological media. It is apparent therefore that the spermatozoa should have a definite pH requirement. Hatziolas[1] has reported a semen pH of 6.89 averaged from 54 bulls. Shergin[2] reports an average pH of 6.74. Jean-Blain (1953) gives a range of 6.20 - 6.95. Herman and Swanson (1941)[3] in their work to establish a correlation between pH and fertility found a pH of 6.0 - 7.0 for bull semen. Emmens (1947) reports a pH range of 7.0 - 8.2, with an average of 7.6 for rabbit semen. Salisbury and Kinney (1957) found the average pH of 9 bull ejaculates to be 6.4.

These findings indicate that there is a considerable variation of pH between semen samples and between species of animals.

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1. Cited after Phillips and Lardy. A yolk pabulum for preservation of bull semen. J.Dairy Sci. 23: 399- 404, 1940.
2. Ibid. p. 399.
3. Cited after Bogdonoff and Shaffner. The effe t of pH on in vitro survival, metabolic activity and fertilizing capacity of chicken semen. Poultry Sci. 33:665, 1954.

The Effect of pH on the Metabolism of Spermatozoa

The effect of pH on the metabolism of spermatozoa, measured by respiration rate, rate of fructolysis, rate of lactic acid accumulation, and motility, has been determined by various workers. Phillips and Lardy (1943) found a definite relation between pH and respiration rate. There was a maximum rate at pH 6.9 - 7.03 and it declined on both the alkaline and acidic sides of this range; at pH 7.5 it was 75% of the maximum. Above pH 7.5 the drop was less pronounced and below pH 6.7 motility dropped very rapidly. In rabbit spermatozoa the optimum pH for motility and respiration was 6.8, and optimum for cock semen respiration was 7.25. Jean-Blain (1953) found that when bull semen was stored at 37°C, the pH and motility dropped with time. This work also showed that the viability of sperms was irremediably affected at pH 6 or less. This drop in pH was attributed to lactic acid accumulation during incubation. These reports indicate that if the pH of the diluter is kept between 6.5 and 7.0 by the addition of egg yolk-phosphate or egg yolk-citrate and stored at 37°C, there is still good viability after 210 minutes. Bogdonoff and Shaffner (1954) working with cock semen found that alkaline pH increases the activity of chicken sperms and acidity reduces it. Motility was best preserved at 7 - 9°C; at a pH of 6.0 minimum activity was obtained as compared with pH of 7.0 and 8.0. Cragle and Salisbury (1957) also noticed that as pH increased in the presence of calcium, the rate of fructolysis and lactic acid accumulation increased in a linear manner, where-

as oxygen uptake responded in a curvilinear form, being low at high and low pH values. Cragle and Salisbury (1959) also found that low pH and high potassium content inhibited all metabolic activities (oxygen uptake, fructose utilization, and lactic acid accumulation) of bull spermatozoa. Emmens (1947) found that rabbit spermatozoa died below pH 5.8. Emmens and Blackshaw (1951) found that ram and bull spermatozoa showed maximum motility at pH 7.0. pH levels of 9.3 to 9.7 hyper- or isotonic media, with partial replacement of NaCl with glucose, were favorable to motility for both species. Flerchinger et al. (1956) reported that high lactic acid accumulation (low pH) was associated with above average maintenance of motility of bull sperms during incubation. Hobbs and Harris (1963) also reported that the motility of chicken sperm was markedly reduced at pH 6.0 and only slightly reduced at pH 7.0. Norman et al. (1958) working with coconut milk and sodium citrate diluters observed that lactic acid accumulated with time and inhibited the metabolic activity of sperms. In a pH range of 5.5 - 5.8, the sperms were maximally inhibited but they still retained their surviving ability. Such sperms upon alkalinization resumed activity and showed 50% motility after 6 days of storage at room temperature. Salisbury and Kinney (1957) studying the effect of pH on the metabolism of bull spermatozoa observed that aerobic fructolysis varied directly with pH, and that carbon dioxide and lactic acid accumulation were highest at high pH levels. In this study the respiration rate measured by oxygen uptake varied considerably from ejaculate to ejaculate thus showing a significant interaction between

ejaculates and pH levels.

These results establish a very fundamental concept regarding the effect of hydrogen-ion concentration on the metabolic activity of spermatozoa. The activity of spermatozoa is generally directly proportional to pH in the range of 5.5 to 7.5 and outside of this range die quickly. Sperms kept in a condition of maximum activity will soon produce enough lactic acid and as a consequence a large drop in pH occurs and inhibits their own activity.

In conventional storage practices the metabolic activity of the sperms is reduced to a low level so that they will not exhaust their energy reserve and will remain alive for a certain length of time. This reduction of metabolism is achieved mainly by lowering the storage temperature. This technique has been employed to its fullest extent in deep freezing of semen, where the sperms are completely inactivated and then reactivated upon thawing prior to insemination.

The observed effect of pH on the sperm activity has suggested the possibility of chemical inactivation of sperms to many research workers. VanDemark and Sharma (1957) at the University of Illinois developped a diluent, which they named Illini Variable Temperature diluent (IVT), which is a modification of egg yolk-citrate saturated with CO_2 thus lowering the pH to 6.35. This experiment indicated that by lowering the pH, inactivation of the sperms was obtained and thus allowed them to be stored at room temperatures. Field results show an average of 75.7% 60 - 90 day non-returns from 111 test cows using semen stored in IVT at room temperature for 1 - 7 days. The corresponding percent non-returns obtained with routinely used 1:3 egg yolk-citrate

was 66.9 in 535 test cows. Willett and Ohms (1958) were successful in inactivating spermatozoa in a yolk-glucose-lactic acid diluter at a pH of 6.4 and reactivating them by raising the pH to 7.0. However inactivation of sperms in yolk-citrate diluter by lowering the pH to 6.4 failed. Eibl et al. (1959) found the optimum pH to be 6.18 - 6.20 for successful inactivation of spermatozoa with CO_2 in a yolk buffer at a storage temperature of 20°C. Barger (1959) found no difference in conception rate between CO_2 inactivated sperms and those stored in citrate buffer within the first 24 hours. In the subsequent 24 hours of storage, conception rate decreased more rapidly in citrate buffer than in CO_2 inactivated samples. Aamdal and Hogset (1960) in two field experiments inseminated 1258 cows with semen stored in egg yolk-citrate for 1 - 3 days, and 1428 cows with semen stored in IVT diluent for 1 - 3 days. Their average non-return percent for both experiments were 64.2 for semen stored in IVT, and 60.2 for semen stored in citrate. Smirnov and Postavnaja (1960) obtained satisfactory field results with IVT diluent. In motility tests no appreciable difference could be found between pH's 6.1, 6.4 and 6.7, during 6 - 7 days of storage. Simunic (1962) obtained 55.3% non-returns on 1186 test cows with semen stored in IVT for 1 - 7 days. The percent of non-returns obtained from semen stored in egg yolk-citrate-glucose control for 1 - 2 days and tested on 210 cows was 66.66. Jaskowski et al. (1962) and Zaljcman (1963) in field trials obtained comparable results with IVT and egg yolk-citrate diluters.

The Effect of pH on Livability in Cold Storage

In 1940 when Phillips and Lardy made their first attempt to preserve bull semen for several days at refrigeration temperatures using egg yolk buffered with inorganic phosphate, it was found that the optimum pH for preserving motility was 6.75 (this average was based upon a rather narrow range of 6.7 - 6.8). The pH range tried was 6.0 - 7.5. Active motility at pH 7.0 fell off rather quickly with time and with a simultaneous drop in pH. At the end of storage period all the pH levels tried dropped to 6.0 - 6.1. It was concluded that the lowered motility was a result of a drop in pH; it was then postulated that a higher pH would delay the time when the critical pH was reached thus prolonging the survival time. This postulation was disproved by experimentation; the addition of fresh egg yolk from time to time to cause a restoration of pH did not prolong survival. Therefore final conclusions were that a restricted pH range in an egg yolk buffer would result in a suitable medium for sperm preservation at 10°C. Anderson[4] used a diluter for ram semen buffered at pH 7.6. In a previous work in 1943 results from an experiment indicated that varying the pH between 6.8 and 7.5 did not greatly affect the activity regardless of preserving ability. In this work it was reported that the motility of cock semen was equally good over a pH range of 6.6 - 7.7, the optimum for maintaining fertility being 7.25. Emmens (1947) found that rabbit semen was more sensitive to acidity than to alkalinity; the best pH for livability being a rather wide range of 7.2 - 7.9.

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4. Phillips and Lardy op. cit. p. 399.

Johnson et al. (1956) working with milk diluters found no appreciable difference in livability among the three pH levels (6.2, 6.6 and 7.0) studied. Rickard et al. (1957) used yolk-citrate-streptomycin-penicillin diluter at pH levels of 7.4, 7.1, 6.8 and 6.5. In these diluters the livability and motility were best preserved at pH 6.5 when stored at 4 - 6°C. However, samples incubated at 37 - 39°C showed better motility at pH 7.4. Wilcox and Shaffner (1957) found very little difference in fertility between pH levels of 6.47 and 7.95 when chicken semen was stored in a purely inorganic phosphate buffer. Motility was found to be a poor measure of fertility when varying levels of pH were used. This work was reconfirmed by Wilcox in 1959 when again the fertilizing ability of cock semen stored in phosphate buffer was shown not to be affected by pH in the levels of 6.5, 7.2 and 7.8. In a recent work Davis and coworkers (1963) studied the livability of bull spermatozoa stored in Tris (hydroxymethyl) aminomethane buffer at 5°C. Of the three levels tested (6.50, 6.75 and 7.00), 6.75 gave the best results.

From this review it seems apparent that pH has some effect on the livability of bull spermatozoa stored at 4-10°C. With other species however (cock and rabbit) pH seems to have no effect on livability in a zone close to neutrality.

MATERIALS AND METHODS

Semen Collection

The experiments were carried out through July and December of 1963. Semen was collected by the use of an artificial vagina from a three year old Holstein-Friesian bull kept at the Agricultural Research and Education Center of the American University of Beirut. The bull was normally used to natural service in a 20 cow herd. Split ejaculate technique was followed whereby each ejaculate was subdivided and subjected to 6 different pH treatments. The livability was assessed by determining the percent alive sperms with direct microscopy in the first, second and fifth day of storage. Ten and five trials were run with egg yolk-citrate and egg yolk-phosphate buffers respectively.

Preparation of Diluters

The citrate buffer was prepared by the proper combinations of 3% sodium citrate dihydrate with 3% citric acid to give average pH values of 7.3, 7.0, 6.8, 6.6, 6.4 and 6.2. The phosphate buffer was prepared by mixing 1% Na_2HPO_4 with 1% NaH_2PO_4 in proper combinations to give average pH values of 7.1, 6.9, 6.7, 6.5, 6.3 and 5.9. All pH's were determined by Radio-meter pH-meter after incorporating 5% fresh egg yolk in the buffers. Stock solutions of citrate and phosphate were prepared 10 - 15 days earlier and fresh egg yolk was mixed a day prior to collection and dilution. All solutions were kept in refrigeration temperatures. The above mentioned salt concentrations were chosen based on the recommendations given by Phillips and Lardy(1940) for the phosphate diluter and Salisbury *et al.*(1941) for the citrate. The egg yolk

however was used in a much lower quantity (5% as compared with 20 - 50% recommended level) which gives a clearer medium and facilitates examination of sperms.

Dilution of Semen

Half a ml. of freshly collected semen was diluted at body temperature in 20 ml. diluent thus giving a dilution rate of 1 to 40. The test tubes were then placed in a beaker of water at body temperature and put in the refrigerator to ensure slow and gradual cooling. Since most of the livability tests were run in Beirut (some 80 kms. away from the collection place) it was necessary to ship the diluted semen. This was done by placing the already cooled samples in a double wall cartoon box along with ice, avoiding direct contact between ice and semen. This method was found satisfactory in keeping the temperature desirably low during the two hour shipment time.

Determination of Live and Dead Spermatozoa

Motility counts and ratings were made by direct microscopy. After shaking the tube gently a drop of diluted semen was placed on a slide previously warmed to approximately body temperature by placing it on the surface of an inverted petri dish placed as a lid on top of a beaker with warm water. The semen was carefully covered with a coverglass to obtain as a uniform film as possible. Ten to fifteen random fields were examined recording the number of live and dead spermatozoa in each one. Percent live sperms were then calculated by averaging the total counts. It was necessary to rewarm the slide from time to time

before a reliable information could be obtained. The sperms also were rated from 0 - 5 according to their speed and vigor of motility, 0 meaning motionless and 5 meaning a vigorous and progressive state of motility. Only good ejaculates were considered, and those which showed an average of 60 percent or less initial motility were discarded.

The collection of semen was usually done at about 10 - 11 o'clock in the morning and the initial motility and rating determined about 5 - 6 o'clock in the evening of the same day. A second and third estimation of motility and rating were done at the end of the second and fifth day of storage at 5 - 10°C.

The artificial vagina and all the other equipment used were simply washed with soap and rinsed with distilled water. In the last two trials of both citrate and phosphate 500 I.U. of penicillin per ml. of diluter were used.

The pH of the diluters were determined both at the beginning of the experiment (without semen) and at the end of storage period (with semen).

RESULTS AND DISCUSSION

The Egg Yolk-Citrate Buffer.

Livability at Different pH Levels

Table 1 and fig. 1 reveal that the average initial motility is not significantly affected by pH in the range of 6.2 - 7.3. This is in contradiction with Phillips and Lardy's work (1943) according to which motility of bull spermatozoa drops very rapidly below pH 6.7. It is also in disagreement with the findings of VanDemark and Sharma who reported that lowering the pH to 6.35 inactivated the sperms. However it confirms the work of Willett and Ohms (1958) who failed to inactivate the sperms in a yolk-citrate diluter at a pH of 6.4.

The percent motility figures show irregular fluctuations with pH in some of the ejaculates. This is due to the uncontrolled temperature at the time of examination. This difference however seems to be eliminated as the number of trials increased as shown by the averages.

The average percent motility in the second day of storage is affected by pH, showing a curvilinear relation (table 1 and figure 1). Maximum livability is obtained at a pH of 6.8. Motility drops on both sides of this value, the drop being more pronounced on the alkaline side. Motility of individual ejaculates seem to respond to the pH in a uniform manner, indicating a rather uniform consistancy. Statistical analyses show that the best pH for survival is within a rather narrow range of 6.6 - 6.8.

At the fifth day of storage this curvilinear relation between pH and motility again holds true (table 1 and figure 1). The maximum

Table 1. Livability of Sperms in Egg Yolk-Citrate Buffer Stored at 5 - 10°C.

pH	Ejaculate Number										Average	
	1	2	3	4	5	6	7	8	9+	10+		
7.3	72	82	75	60	75	68	75	75	88	80	75	Initial motility %
7.0	76	85	73	67	70	79	78	68	88	78	76	
6.8	84	71	70	60	70	85	82	69	84	85	76	
6.6	78	79	69	63	61	78	82	65	86	78	74	
6.4	80	78	66	66	64	78	82	59	83	77	73	
6.2	78	78	72	68	73	77	79	70	87	81	76	
Average	78	79	71	64	69	78	80	68	86	80		
7.3	23	11	53	43	22	42	48	46	61	58	41	2nd day motility %
7.0	55	40	38	34	38	60	53	59	65	67	51	
6.8	53	55	57	53	50	71	66	69	74	69	62	
6.6	34	59	47	27	44	80	67	75	82	75	59	
6.4	47	48	44	42	42	75	72	60	80	74	58	
6.2	44	25	47	3	36	70	68	66	79	71	51	
Average	43	40	48	34	39	66	62	62	74	69		
7.3	8	8	12	3	19	40	5	27	36	2	16	5th day motility %
7.0	10	5	21	8	25	50	18	21	65	1	22	
6.8	15	24	8	12	34	50	32	42	65	46	33	
6.6	14	25	25	4	42	48	36	39	60	48	34	
6.4	12	6	6	20	38	50	41	29	50	21	27	
6.2	16	5	5	5	33	42	56	22	40	23	25	
Average	13	12	13	9	32	47	31	30	53	24		

+ 9 and 10 are the same ejaculate, No. 10 is diluted after shipment.

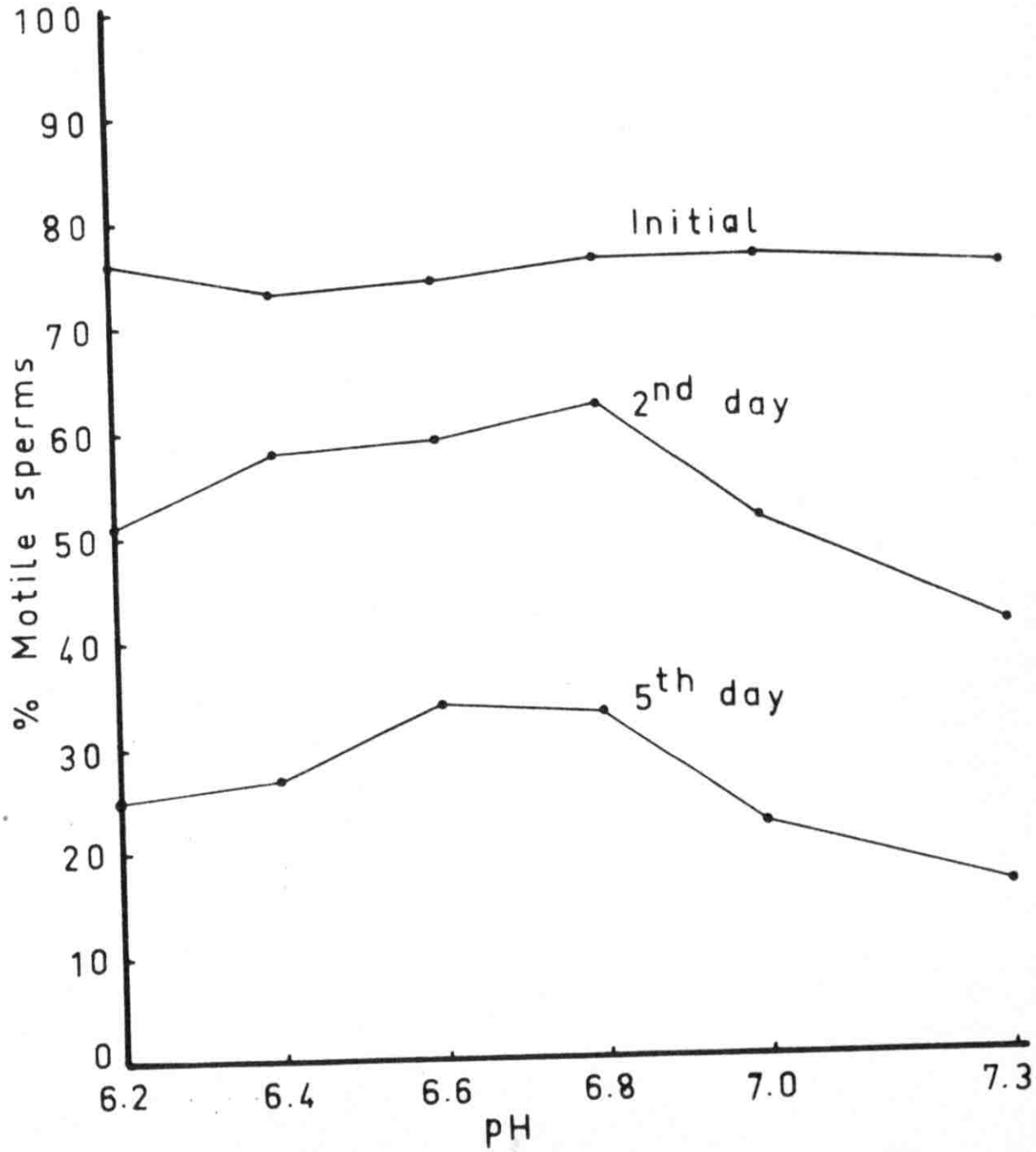

Figure 1. Livability of bull spermatozoa stored in Egg yolk-citrate buffer at different pH levels and at 5-10°C for 5 days. (Average of 10 trials).

motility in this case seems to be in a range (6.6 - 6.8), livability tapering on both sides of this range with a faster rate on the higher pH values. The differences between pH are statistically significant. It can be concluded that bull spermatozoa retain their motility better in the pH range of 6.6 to 6.8 in egg yolk-citrate buffer when stored at 5-10°C. This result is in fairly close agreement with Rickard et al. (1957) who found the best pH for preserving the motility in a yolk-citrate diluter at 4 - 6°C to be 6.5.

Livability of Different Ejaculates

Table 1 shows a considerable variation in initial percent motility between ejaculates. The average percent motility range between 64 and 86% with an average of 75. The statistical difference between ejaculates was highly significant at 1% level. This difference can partly be attributed to the frequency of ejaculation and partly to the degree of sexual stimulation of the bull. The more the bull was excited at the time of collection (either by the presence of a cow in heat or by few false mounts) the better was the ejaculate. Such ejaculates had both a thick and creamy consistancy when undiluted and a high percent of live sperms. On the other hand, when the bull was less excited the ejaculate was thinner, mainly being seminal fluids, had a lower percent live sperms, and the volume was smaller. In general the bull showed less libido in serving the artificial vagina, since he was normally used for natural mating.

The second and fifth day average motility figures show a still wider range between ejaculates. These ranges were 34 to 74% for the second,

and 9 to 53% for the fifth day storage. The differences were highly significant at 1% level. These results indicate that ejaculates with a high initial motility percent will keep their activity much better in cold storage than ejaculates with low initial motility.

Activity Rating

The activity of the spermatozoa was rated according to the following scheme:

Activity rating	State of Spermatozoa
4 - 5	Full activity with vigorous progressive motility. There may be up to 30-40% dead sperms.
3 - 4	Cells active but the progressive motility rather sluggish.
2 - 3	About 1/3 of motile sperms show a fairly progressive motility, others only oscillating.
1 - 2	Very few cells exhibit slow progressive motility, others showing oscillatory movements, many with only tails vibrating.
0 - 1	Almost all cells stationary with their tails vibrating. Occasionally some showing a poor progressive motility.
0	All cells dead.

Table 2 gives the activity ratings of sperms in the citrate buffer. It appears that neither pH nor the ejaculate have affected the

degree of activity of the spermatozoa. Here the effect of pH on sperm activity is similar to its effect on percent motility, while ejaculates showing low percent motility have as good a motility rating as those with high percent motility. A rather indirect explanation for this could be given. It is generally true that percent motility alone is not a sound criterion to evaluate a semen sample especially when the motility is above 50 - 60%, because the correlation between percent motility and conception rate is rather low. This means that two ejaculates of different percent motilities (provided that they are above 50 - 60%) could produce equally good conception rates. It is likely therefore that ejaculates with low percent motility could have sperms of high activity rating which is indicated in the present experiment.

The average ratings on the second day of storage differ more between ejaculates rather than between pH levels. This indicates that the sperms in ejaculates of high initial motility percent have a higher surviving power. This is also revealed in the fifth day ratings where a difference between pH values also exists indicating that the pH started to show an effect on rating after the second day of storage.

pH Drop During Storage

Table 3 shows a drop in pH in the citrate buffer. The drop is greatest in the highest pH value and it decreases with decrease in pH. This drop could be attributed to two causes:

1. The metabolic activity of the spermatozoa, although very low at refrigertion temperature may be high enough to produce a sufficient amount of lactic acid to cause this drop.

Table 2. Activity Rating of Sperms in Egg Yolk-Citrate Buffer Stored at 5 - 10°C+.

pH	Ejaculate Number										Average	
	1	2	3	4	5	6	7	8	9	10		
7.3	5	5	5	5	5	4	5	5	5	5	4.9	Initial rating
7.0	5	5	5	5	5	5	5	5	5	5	5	
6.8	5	5	5	5	5	5	5	5	5	5	5	
6.6	5	5	5	5	5	5	5	5	5	5	5	
6.4	5	5	5	5	5	5	5	4.5	5	5	4.9	
6.2	5	5	5	5	5	5	5	5	5	5	5	
Average	5	5	5	5	5	4.8	5	4.9	5	5		
7.3	3	1.5	3	4	1	.5	2	3	4	4	2.6	2nd day rating
7.0	3	3	3	3	3	1.5	4	3	4.5	4.5	3.6	
6.8	4.5	3	3	5	3	2.5	4	4.5	4.5	4.5	3.9	
6.6	3	3	3	3	3	3	5	5	5	4.5	3.8	
6.4	3	2.5	2	4	3	3	5	4.5	4.5	4.5	3.6	
6.2	3	2	3	1	2	3	5	5	4.5	3.5	3.2	
Average	3.3	2.5	2.8	3.3	2.5	2.3	4.2	4.2	4.5	4.3		
7.3	0.5	0.1	1	0.5	1	0.5	0.5	2	3	0.1	1	5th day rating
7.0	0.2	0.1	1.5	0.1	1	1	1	1	3.5	0.1	1	
6.8	0.2	2	1	0.5	1	2	2	4	4	3	2	
6.6	1	1.5	2	1	3	3	2	3	4	3	2.4	
6.4	0.2	0.5	0.1	0.1	3	3	3	3	4	1	1.8	
6.2	0.2	0.2	0.1	0.1	2	2	3	1	3	1	1.3	
Average	0.4	0.7	1	0.4	2	2	2	2.3	3.6	1.4		

+ 0 represents no activity

5 represents vigorous progressive motility

Table 3. Drop in pH during 5 day cold storage period in Citrate and Phosphate buffers.

Egg Yolk-Citrate buffer		Egg Yolk-Phosphate buffer	
pH		pH	
Initial	Final	Initial	Final
7.3	6.8	7.1	7.0
7.0	6.6	6.9	6.8
6.8	6.6	6.7	6.6
6.6	6.4	6.5	6.5
6.4	6.3	6.3	6.2
6.2	6.3	5.9	5.8

2. The weak buffering capacity of the solution specially at the higher pH range employed (Britton 1932) can be considered a second cause for the pH drop. This reason seems to be the more important, since as we saw before, the activity of the sperms was not affected very much by pH and hence the amount of lactic acid produced could not be more at the higher pH levels than at the lower.

The egg yolk-phosphate buffer

Livability at Different pH Levels

The effect of pH on the initial motility does not show any consistency (table 4 and figure 2). The fluctuations are statistically significant. A higher number of trials could probably reduce this fluctuation. The only five trials in the phosphate buffer, in which the examination of sperms was particularly difficult (and hence less accurate) due to flocculation of fat from the yolk, looks to be insufficient for a difinite conclusion.

The second day motility results are definitely affected by pH showing a very highly significant difference between pH (table 4 and figure 2). This high significant difference is caused by the very low pH's employed (5.9 - 6.3) which checked the motility considerably. The other levels of pH (6.5 - 7.1) do not differ as much although there seems to be an optimum at 6.5 - 6.9 level since the motility starts to drop above 6.9.

The fifth day motility results follow the same general pattern as the second day with regard to pH, meaning that the influence of pH on the

Table 4. Livability of Sperms in Egg Yolk-Phosphate Buffer Stored at 5 - 10°C.

pH	Ejaculate Number+						
	1	2	3	4	5	Average	
7.1	56	65	67	76	76	68	Initial motility %
6.9	71	67	65	79	74	71	
6.7	57	65	69	76	74	68	
6.5	68	70	75	78	73	73	
6.3	60	66	62	75	67	66	
5.9	58	55	57	72	72	63	
Average	62	65	66	76	73		
7.1	34	52	50	43	50	46	2nd day motility %
6.9	39	59	46	56	61	52	
6.7	37	64	35	67	56	52	
6.5	44	60	37	55	52	50	
6.3	20	42	33	44	39	36	
5.9	2	2	7	3	9	5	
Average	29	47	35	45	45		
7.1	0	10	3	13	0	5	5th day motility %
6.9	5	9	0	14	3	6	
6.7	6	10	0	21	3	8	
6.5	4	2	2	27	3	8	
6.3	0	00	0	10	5	3	
5.9	0	0	0	0	0	0	
Average	3	5	1	14	2		

+ Ejaculates 1, 2, 3, 4 and 5 correspond to ejaculates 6, 7, 8, 9 and 10 respectively in table 1.

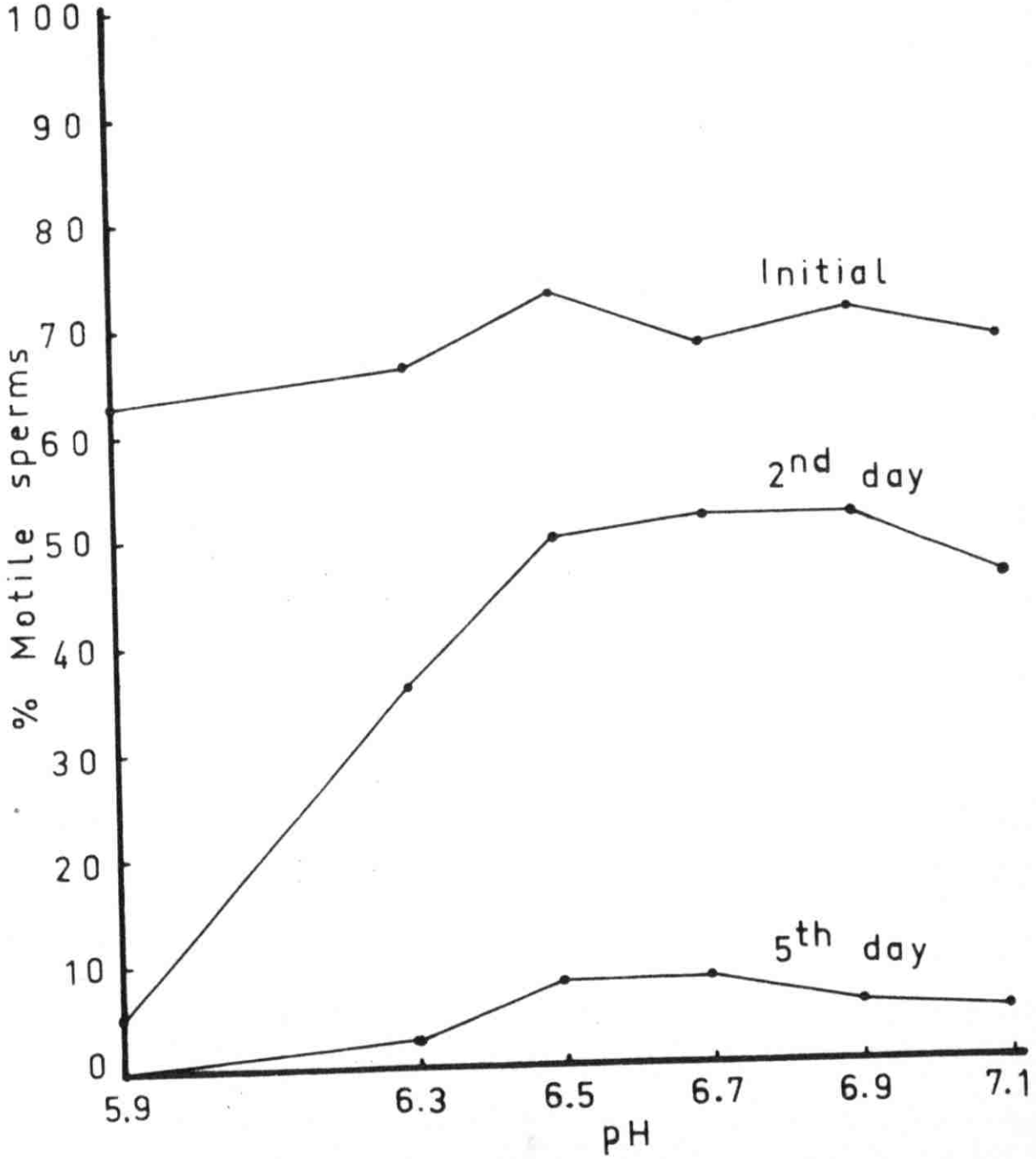

Figure 2. Livability of bull spermatozoa stored in Egg yolk-phosphate buffer at different pH levels and at 5-10°C for 5 days. (Average of 5 trials).

livability is rather uniform all through the 5 days of storage. There was however no significant difference between pH levels.

Livability of Different Ejaculates

In the case of phosphate buffer the fluctuation of the initial percent motility figures between ejaculates is less than it was in the case of citrate, although the differences are statistically significant at 1% level. Here again the differences between ejaculates can be explained the same way as in the citrate, because the ejaculates are identical.

On the second and fifth day of storage we find statistically significant difference between ejaculates with the one with the highest initial live sperm count, having the highest final motility percent in conformity with the citrate buffer.

Activity Rating

Table 5 shows the activity rating of sperms in the phosphate buffer. The pH seems to have no effect on the activity of sperms except at the extreme level on the fifth day and only at the lowest pH's on the second day rating. The effect of pH on activity rating follows the same trend as its effect on percent motility in this buffer. No definite conclusion can be drawn from the figures in table 4 regarding the effect of ejaculate on the activity rating. It appears to be proportional to percent motility.

pH Drop During Storage

Table 3 shows a negligible but consistant drop in pH in the

Table 5. Activity Rating of Sperms in Egg Yolk-Phosphate Buffer Stored at 5 - 10°C+.

pH	Ejaculate Number						
	1	2	3	4	5	Average	
7.1	3	5	4	4.5	4.5	4.2	Initial rating
6.9	3	5	4	5	4.5	4.3	
6.7	3	5	4	4.5	4.5	4.2	
6.5	3	5	5	5	4.5	4.5	
6.3	4	5	4	4.5	4	4.3	
5.9	3	5	4	4.5	4	4.1	
Average	3.2	5	4.2	4.7	4.3		
7.1	2	4	3	3	3	3	2nd day rating
6.9	2	4	2	3	3.5	2.9	
6.7	1.5	4	2	4	3.5	3	
6.5	2	4	2	3.5	3	2.9	
6.3	1	3	2	3	3	2.4	
5.9	0.5	1	1	0.1	0.5	0.6	
Average	1.5	3.3	2	2.8	2.8		
7.1	0	1	0.1	1.5	0	0.5	5th day rating
6.9	0.5	1	0	1.5	0.1	0.6	
6.7	0.5	1	0	2.5	0.1	0.8	
6.5	0.5	0.1	0.1	3	0.1	0.8	
6.3	0	0	0	1	0.1	0.2	
5.9	0	0	0	0	0	0	
Average	0.3	0.5	0	1.6	0.1		

+ 0 represents no activity

5 represents vigorous progressive mobility.

phosphate buffer. A generally lower sperm activity and a very high buffering capacity of the sodium dihydrogen and disodium hydrogen phosphate mixture account for this small drop in pH. The latter being more effective than the first, specially in explaining the consistancy in the drop, since the phosphate has its maximum buffering capacity in the range of 5.91 - 8.04 (Britton 1932).

Comparison Between Buffers

Some minor differences between the citrate and phosphate buffers have already been mentioned above. Here are few more general and fundamental differences:

1. The citrate buffer used in this experiment was a better medium for storing bull semen than the phosphate, when 5% egg yolk was used in both buffers.
2. The phosphate buffer had a better buffering capacity than the citrate in the studied pH range.
3. It was far easier to examine the spermatozoa under the microscope in the citrate than in the phosphate buffer, since the former had the ability of dispersing the fat globules in the medium thus producing a clearer microscopic field.
4. Precipitation of solids occured more in the phosphate buffer, specially at low pH range.

SUMMARY AND CONCLUSION

1. The effect of hydrogen-ion concentration on the livability of bull spermatozoa, stored in egg yolk-citrate and egg yolk-phosphate at refrigeration temperatures, was studied.
2. Split ejaculate technique was employed whereby ten trials with nine ejaculates, and five trials with four ejaculates were conducted for citrate and phosphate buffers, respectively.
3. Six portions, each of 0.5 mls., were drawn from each ejaculate and subjected to six different pH treatments in 6 test tubes of 20 mls. each.
4. The pH was varied by suitable combinations of sodium citrate dihydrate and citric acid in one case, and disodium hydrogen and sodium dihydrogen phosphate in the other. The pH levels were 6.2, 6.4, 6.6, 6.8, 7.0 and 7.3 in the citrate and 5.9, 6.3, 6.5, 6.7, 6.9 and 7.1 in the phosphate buffers. The pH's were determined by a Radiometer pH-meter after the addition of 5% egg yolk in the buffers.
5. The livability was assessed by determining the live and dead sperms on the day of collection and after the two and five days of storage by direct microscopic count.
6. The pH affected the livability of bull spermatozoa in both diluents under the employed conditions. Its effect however is only slight within the ranges of 6.6 - 6.8 and 6.5 - 6.9 for the citfate, and the phosphate buffers, respectively.
7. The livability was better in the citrate buffer than in the phosphate.

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