Optimizing stallion semen dilution based on hydrogen ion index

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Abstract. The dependence of the results on the individual qualities of stallion semen is very high, which is an obstacle to the wider use of the cryopreservation method in practice. It has been shown in this work that semen from successive samples from the same stallion has different hydrogen ion concentrations (pH) and different buffer capacities. When Lactose-chelate-citrate-yalk environment (LCHCY) is diluted, the pH of semen varies depending on its initial value. But in 30-40% of cases, the hydrogen ion concentration (pH) shifts more or less from the expected value. In order to establish the maximum possible survival time of refrigerated and cryopreserved semen, the optimum pH acidity value after dilution was determined. The maximum survival time depended on the initial sperm concentration. It was possible to adjust the acidic pH value after dilution by dropwise addition of buffer solutions of the initial medium to the acidic or alkaline side. An individual approach to the semen dilution procedure by adjusting the pH value after dilution makes it possible to increase the number of stallions for artificial insemination with semen resistant to refrigeration and cryopreservation.

1 Introduction

Despite the development of methods for freezing sperm in mini-volumes (1 and 5 ml), the method of cryopreservation in the volume of 15-20 ml is still used in our country. This method has proven itself in practice due to its simplicity, inexpensive equipment and stable results. The Russian technology of cryopreservation in large volumes has shown high safety and efficiency of using chilled and frozen-thawed seed. At present, progeny after long periods of seed storage in liquid nitrogen (37-50 years) without reduction of fertilising ability have been obtained [1].

The technology for preparing semen for freezing in large volumes is gentle on the sex cells, without the use of centrifugation. This makes it possible to use desirable stallions with semen of poor quality. A sufficient number of motile sperm in a dose is 150-200 million. In other words, an economical consumption of genetic material is achieved due to the volume consisting of 75 % of the medium.

Application of cryopreservation technology is carried out using lactose-chelate-citrate-yolk (LCHCY) (LCCY-lactose-chelate-citrate-yolk) medium. The introduction of acid-salt

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buffer consisting of chelato-citrate-bicarbonate complex into the diluent allowed to prolong the storage time of semen in the refrigerated state and to increase the level of gestation of frozen semen to the level of natural breeding [2]. When diluted with this medium, sperm stability was significantly affected by hydrogen ion concentration (pH). LCHCY medium changes the alkaline reaction of freshly obtained sperm to a slightly acidic reaction, which favours the transition of sperm to a low-active state, thus preserving their vital resource. In addition, the acidic buffer of this medium neutralises the influence of harmful secretions of the accessory sex glands without removing them, i.e., centrifugation is not necessary.

But not all stallions have good sperm preservation and fertilisability. The dependence of the results on the individual qualities of the semen is very high, which is an obstacle to the wider use of cryopreservation in practice. Many researchers find the most significant individual characteristics of stallion semen within breeds rather than between breeds [3].

The general health status of the sire's organism may affect semen quality and its cryostability. In stallions with decreased sperm motility and survival rates, an increase in eosinophils, a-amylase and urea [4] and fibrinogen [5] levels in the blood was found. The presence of pathological processes in the organism of stallions leads to a decrease in sperm quality.

It is important to remember the importance of adequate feeding and motility, which influence the quality of sperm production. Any unbalanced diet has a negative effect on all stages of spermatogenesis and the subsequent quality of the sperm produced.

Differences in artificial insemination performance as well as semen cryostability are evident at any age, but the highest percentage of stallions with poor semen quality after freeze-thawing in the older sire group is 54 % [6]. The reserve of antioxidant capabilities of semen plasma and the ability to resist oxidative stress in young stallions is 2.1 times higher than in older stallions, notes M.M. Atroshchenko et al. (2022) [7-8].

The secretion of quality semen from stallions is influenced by seasonal factor while affecting the hormonal and enzyme levels of blood and semen [9-10]. R.L. Senra et.al. (2022) noted that during the breeding season there was an increase in different types of enzymes, an increase in semen volume and an increase in glucose and cholesterol concentrations [11].

Removing plasma after centrifugation of sperm is considered a useful technique for long-term sperm preservation and cryopreservation. But experiments on plasma removal after sperm centrifugation have yielded contradictory results. Several studies have shown a favourable effect of sperm plasma removal on sperm motility [12-13]

But the effects of sperm plasma removal depend on the choice of techniques, diluents that vary in quality and quantity, osmolarity and pH [13]. Because of the inherent variability in stallions, it may be useful to modify semen handling procedures for stallions producing semen with low storage stability or with toxic plasma, as recommended by J. A. Len et.al. (2010) [14]. Successful experiments on the replacement of sperm plasma from one stallion with good motility for the semen of another stallion with poor motility, after removal of his own sperm plasma, are presented.

A. Andrade [15] notes that the response to seminal plasma regarding sperm preservation is different for each ejaculate. Substances have been identified in seminal plasma that decrease or increase the fertilisation capacity of spermatozoa. Very often thawed semen reacts favourably to seminal plasma added to the thawing medium.

Many authors in their studies show the influence of seminal plasma on the quality of fresh and thawed semen of stallions [16-18]. Plasma contains substances necessary to maintain the viability and fertility of spermatozoa, but its excess can negatively affect the preservation of sperm.

It is believed that obtaining semen in a fractional manner makes it possible to preserve only the dense part of the ejaculate and this has a positive effect on its preservation. M.A.

Kareskoski (2011) [19] studied the composition of sperm-rich fractions and sperm-poor fractions and found differences in composition. The sperm-rich fractions contained the highest levels of proteins, alkaline phosphatase, acid phosphatase and glucuronidase enzymes. The sperm-poor fractions contained the highest concentrations of calcium and magnesium. The highest levels of sodium and chloride were found in the pre-sperm fluid.

Sperm survival is influenced by many components of seminal plasma, and in different directions: some positively and others negatively due to its complex composition and heterogeneous secretion even in the same stallion [20]. Freshly obtained semen with the same pH values changes its readings differently after dilution, which depends to a large extent on its buffering properties [21].

The presence of weak acid salts of carbonates, citrates, lactates and phosphates in semen plasma determines its buffering stability. A strong buffer of semen is salts of citric acid. Semen proteins increase the buffering capacity of semen as they are able to bind both acidic and alkaline substances. Buffering plays a major role in ensuring the viability of sperm, protecting them from damage associated with sudden changes in the environment. Depending on which elements predominate in the ejaculate, pH changes occur after dilution.

An example of consecutive semen samples from one stallion showed very large quantitative variations of components within the range reaching 10-25 multiple differences [20]. Thus, the value of minimum and maximum quantities in seminal plasma of the same sire differed in the content of the enzyme creatine phosphokinase (CPK) by a factor of 9, lactic dehydrogenase (LDH) 25-fold, acid phosphatase 10-fold, glucose 7-fold, lactate 14-fold.

Researchers of the American company Select Breeders Services (SBS) proposed to dilute stallion semen with different variants of Spectrum medium depending on the individual qualities of semen, which are determined by testing with a special test solution, the composition of which is patented [22]. A range of Spectrum media from SBS brand Spectrum has appeared on the Russian market: Spectrum Red, Spectrum Orange, Spectrum Violet, Spectrum Blue, Spectrum Green. It is reported that the use of different diluent formulations has resulted in a much higher percentage of stallions whose semen withstands freezing.

In our practice, we have accumulated observations that pH changes after semen dilution with the same medium do not occur in the same way in different stallions and even in different ejaculates from the same stallions. This leads to uneven results of sperm preservation during cooling and cryopreservation.

In order to prolong the storage time of sperm in the chilled and frozen state, the task was set to establish the optimal value of the hydrogen ion index (pH) of semen after dilution and to determine the possibility of correcting the pH of diluted semen.

2 Methods and materials

In the experiment 60 ejaculates from 7 stallions of the experimental stables of the All-Russian Research Institute for Horse Breeding were used. The stallions were used for semen collection on an artificial vagina in a constant mode with a sequence of 2 times a week. The feeding ration was standard for all stallions. After collection and filtration, native semen was evaluated for volume, concentration, total sperm count, motility, and acidity (pH). A computerised CASA score was used to determine sperm motility.

Sperm was then diluted with LCHCY medium according to its concentration. If the value was less than 200 million/ml, the dilution was 1:2, if the value was 200-400 million/ml, the dilution was 1:3, and if the value was more than 400 million/ml, the dilution was 1:4. One part of the diluted semen was left for storage chilled in a household

refrigerator, the other part was frozen in liquid nitrogen, according to the 'Instruction on artificial insemination and transplantation of equine embryos'. After thawing, sperm motility and survival rate were determined in hours when semen was kept in the refrigerator at the temperature 2-4℃.

To determine the hydrogen ion concentration (pH) of fresh and diluted semen, the pH-meter-ionometer 'Expert-001' with a combined electrode was used (Scientific and Production Enterprise "Econix-Expert", Russia).

For a more complete judgement of the acid-alkaline balance, the alkaline and acid buffering capacity of fresh semen was determined by titration according to standard methods. The alkaline buffering capacity (acid components) was determined by titration in ml with caustic sodium NaOH (0.01 n p-p), Acid buffer capacity (alkaline components) - by hydrochloric acid titration HC1 (0.01 n p-p).

The optimal pH value of diluted semen was determined by a series of dilutions in the range of pH values from 6.0 to 7.0 (with an interval of 0.1-0.2) by adjusting the diluent with additions of sodium bicarbonate (included in the medium) and then determining the maximum survival rate of diluted semen variants.

Further statistical processing of the results was carried out using Microsoft Excel and Statistica 10 programme.

3 Results

Continuous measurements of hydrogen ion concentration in fresh semen and after semen dilution revealed fluctuations within wide ranges. In our experiments, freshly obtained semen of stallions had pH fluctuations in the range of 7.0-7.9 with an average of 7.45. The concentration of hydrogen ions LCHCY of the medium was in the range of 6.1.

Figure 1 shows an example of how the hydrogen ion concentration (pH) and the acid and alkaline buffer change during successive semen collections from Crystal. These readings were different at each successive collection. In addition, at the same pH value (option 1 and 2 in Figure 1), the acid and alkaline buffering properties of native semen were different.

In data processing (for 60 ejaculates), the pH of native semen had a correlative positive relationship with sperm concentration (0.4) and a negative relationship with ejaculate volume (-0.29).

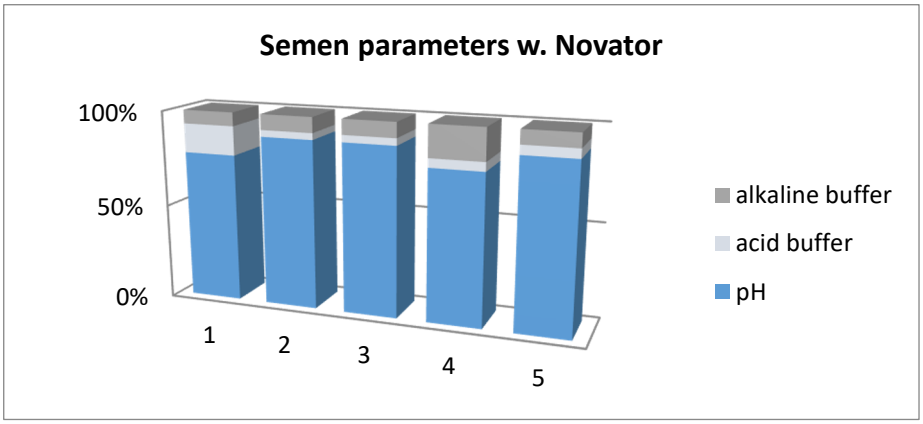


Fig. 1. pH values of native semen, acid and alkaline buffer at consecutive semen collections from the stallion Crystal.

Table 1 and Figure 2 show that the pH changes after diluent application are different, but depend on the initial ejaculate pH value. On average, a pattern can be observed: at higher pH of semen, the shift after dilution is more significant, i.e. the higher the alkalinity (pH 7.56-7.9), the more the pH shifts from the initial value to the acidic side. At the same time, more individual variations of this indicator in one or the other direction are detected. These deviations, which occurred in 30-40% of cases, made us conduct experiments to establish the optimal pH value of diluted semen. The pH value at which sperm motility and survival time are maximised was determined. Differences in sperm concentration and, accordingly, different degrees of dilution led to different results in terms of maximum motility and survival time.

Table 1. pH changes during semen dilution process (n=60) at different initial values of hydrogen ion concentration.

Variant No.	native sperm pH	sperm after dilution pH	oscillation limits (min-max)
1	7.01-7.24	6.00-6.51	0.57-1.17
2	7.25-7.55	6.10-6.60	0.74-1.37
3	7.56-7.75	6.12-6.66	0.80-1.66
4	7.76-7.90	6.15-6.85	0.92-1.72

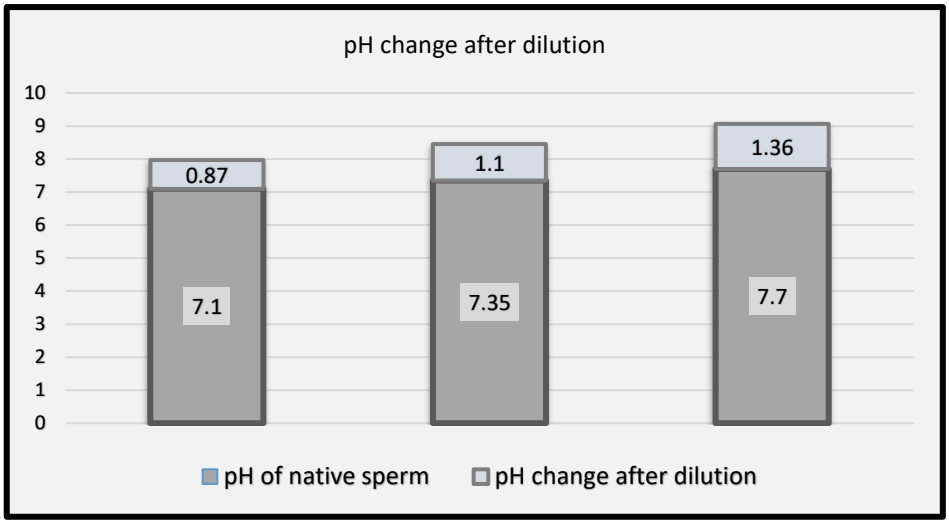
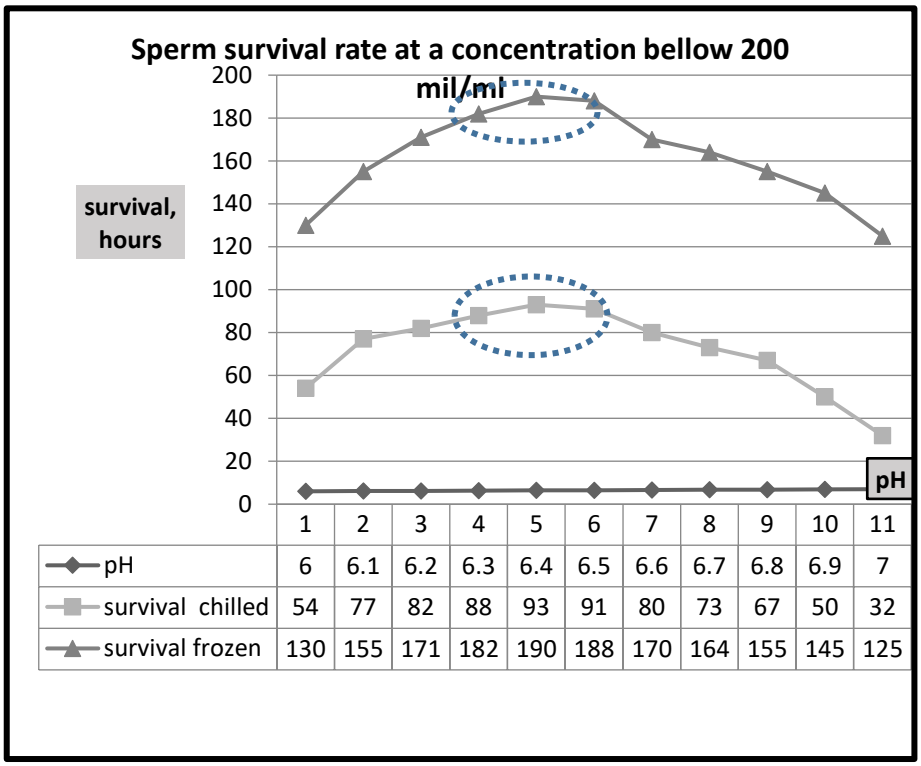


Fig. 2. Diagram of the average pH change during semen dilution at different pH values of native semen.

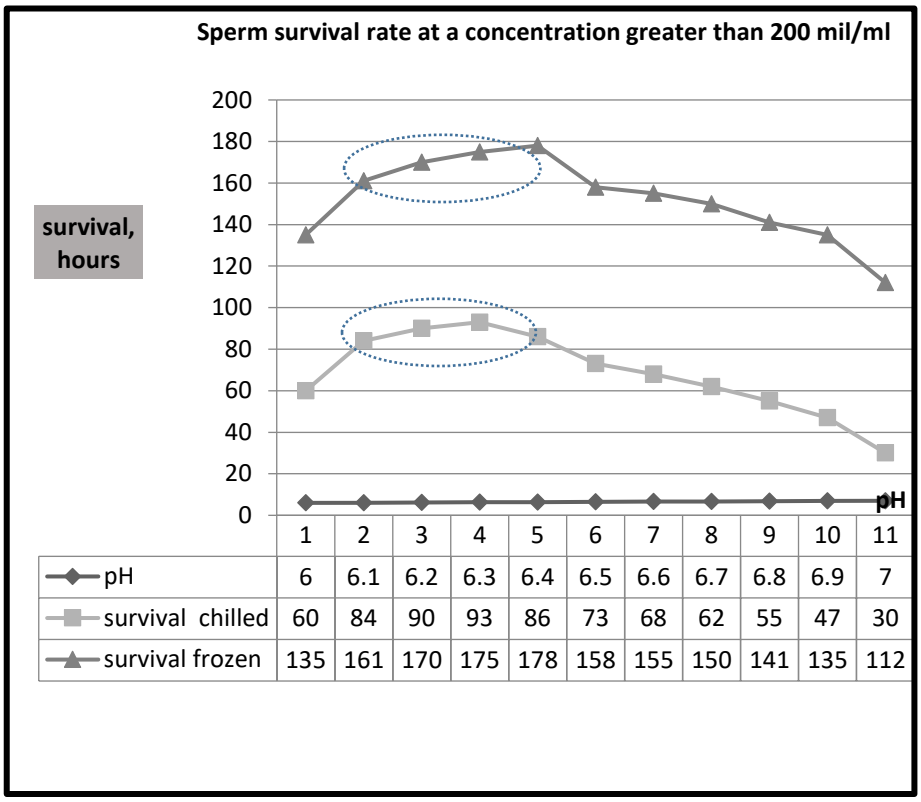
When sperm concentration was equal to or less than 200 million/ml (1:2 dilution of semen with medium), the optimum pH ranged from 6.3-6.5 (Figure 2). At sperm concentrations greater than 200 million/ml (1:3 and 1:4 dilution), the optimum pH of diluted semen ranged from 6.1-6.4 (Figure 3). In all cases, the motility and survival rates of semen diluted to optimum pH values were higher by 5 - 20%, compared to other pH values. Total sperm motility readings after thawing for the optimal variants ranged from 2.7-3.0 points, in contrast to the borderline variants of 2.0-2.3 points. Figures 3 and 4 show the determination of optimum pH values for survival rate, where the graph clearly shows the

maximum values of this index for chilled and freeze-thawed semen. In borderline variants the difference is reliable ($p < 0.05$), the difference is highly significant in the extreme variants ($p < 0.01$).



	1	2	3	4	5	6	7	8	9	10	11
pH	6	6.1	6.2	6.3	6.4	6.5	6.6	6.7	6.8	6.9	7
survival chilled	54	77	82	88	93	91	80	73	67	50	32
survival frozen	130	155	171	182	190	188	170	164	155	145	125

Fig. 3. Sperm survival in chilled and thawed semen for different pH values at a concentration of 100-200 million/ml and 1:2 dilution with medium.



	1	2	3	4	5	6	7	8	9	10	11
pH	6	6.1	6.2	6.3	6.4	6.5	6.6	6.7	6.8	6.9	7
survival chilled	60	84	90	93	86	73	68	62	55	47	30
survival frozen	135	161	170	175	178	158	155	150	141	135	112

Fig. 4. Survival of chilled and thawed semen at different pH values at concentrations >200 million/ml and dilution with 1:3 to 1:4 medium.

Establishment of optimal pH values of diluted semen made it possible to individually adjust the acidity of semen, bringing its pH to the optimum, which can be regulated by adding buffer components included in the LCHCY-medium: sodium bicarbonate (alkaline component) or chelaton (acidic component).

Experimentally, it has been found that a pH shift of 0.1 unit usually requires no more than 0.1 ml of one of the buffer components. Therefore, this method is readily feasible, provided that a pH measuring instrument is available.

4 Discussion

As a result of experiments with constant measurement, it was confirmed that when LCHCY medium is diluted with semen, the pH does not always change to the expected value. In 30-40 % of cases, the acidity (pH) shifts more or less from the expected value. We believe that this is due to the ingestion of secretions of the genital glands and sperm in different proportions in the ejaculate. The data of Figure 1, which shows the uneven penetration of acidic and alkaline components even at the same pH, confirm this conclusion. The uneven penetration of different plasma components into the ejaculate has been noted by many authors (19-21) and the need to select a suitable diluent in case of deviations in the values of.

We were faced with the task of correcting the acidity of diluted semen in the event of a pH shift to one side or the other in order to neutralise the excess of elements. Practice has

shown that such an experiment is possible by changing the proportions of buffer components included in the medium.

Experiments to establish optimal pH values in diluted semen are not described in the literature, although optimal pH values of diluents are indicated. Testing a wide range of pH values made it possible to determine the optimum pH value in semen diluted with LCHCY-medium at which sperm survival is maximised. For different sperm concentrations, the pH optimum is slightly different due to differences in the degree of dilution. As a result, sperm motility and survival rate at optimum pH values, was significantly higher compared to other values.

The condition for practitioners to have an additional inexpensive pH measuring device is easily realisable. This will allow for individualisation of the semen dilution process and correction of the final pH, reduce the number of spermodose rejections and increase the percentage of stallions for artificial insemination with chilled and frozen semen.

5 Conclusions

1. Studies have shown that individual variations in the acidity of semen, and its buffering elements lead to uneven results after dilution.

2. The optimal value of the hydrogen ion index in stallion semen after dilution with LCHCY-medium has been established, at which sperm motility and survival rate are at the maximum level.

3. A personalised approach to the procedure of stallion semen dilution by adjusting the pH after dilution with buffer components of the medium has been proposed.

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