The role of sodium bicarbonate for the buffering capacity of semen and for the receptivity of cervical mucus for sperm

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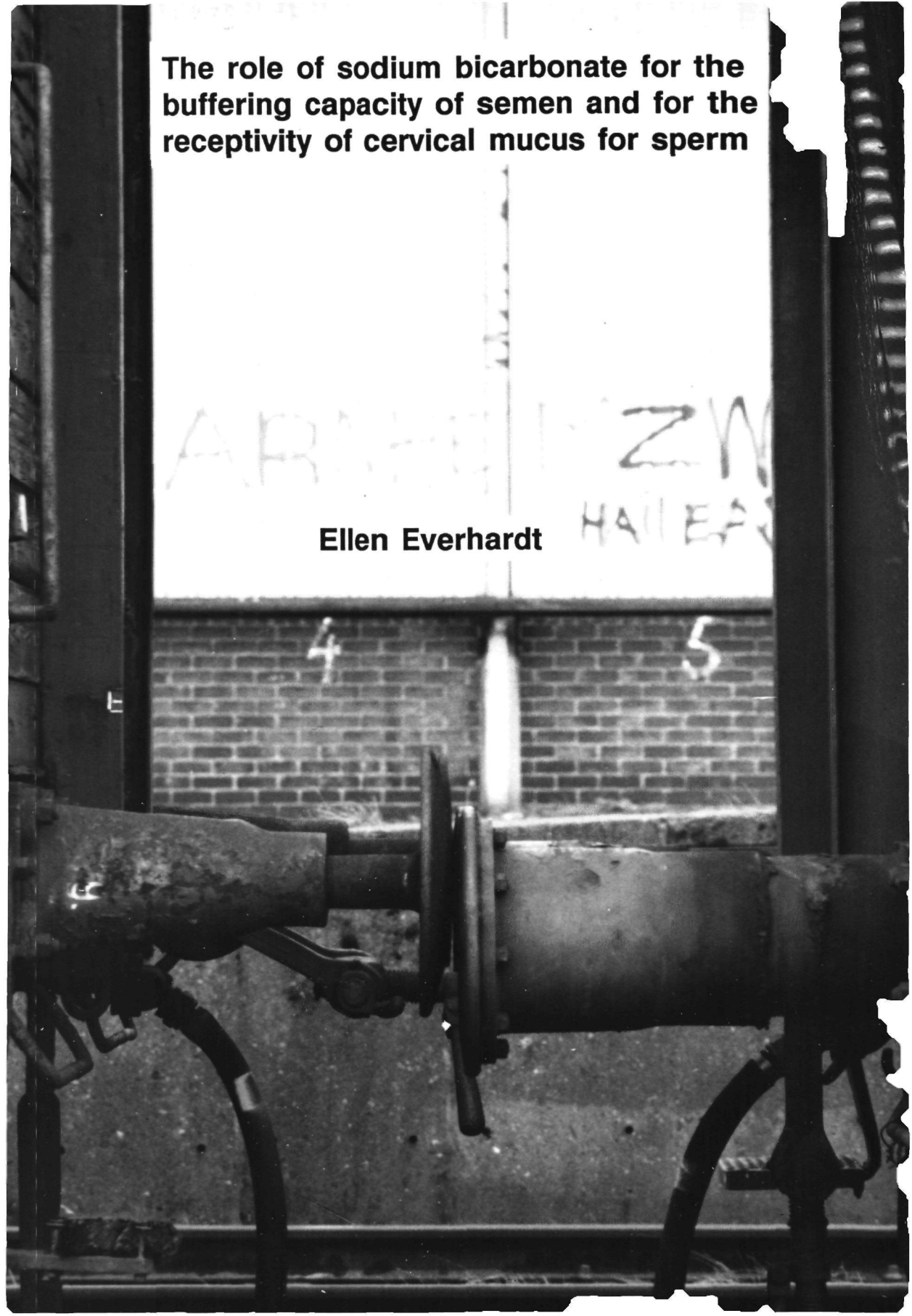

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een wetenschappelijke proeve op het gebied van de
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Aan Rob en Tom

Aan mijn vader en moeder

'desondanks'

ABBREVIATIONS

AIH	: arteficial insemination husband
AID	: arteficial insemination donor
BBT	: basal body temperature
BC	: buffering capacity
CD	: day of cycle
EE	: ethinyl estradiol
GIFT	: gamete intra falopian transfer
hCG	: human chorionic gonadotropin
HMG	: human menopausal gonadotropin
HPF	: high power field
HVC	: high viscosity component
IVF	: in vitro fertilization
LH	: luteinizing hormone
LH-SIR	: luteinizing hormone surge initiating rise
LVC	: low viscosity component
mIU	: milli international units
n	: number of subjects
OSM	: osmolality = measure of the total number of particles in the solution
P	: * progesterone * level of significance
PCT	: post coital test
SD	: standard deviation
SEM	: * standard error of the mean * scanning electron microscopy
SPT	: sperm penetration test
WHO	: world health organization
wt/vol	: weight/volume
ZIFT	: zygote intra falopian transfer

CONTENTS

INTRODUCTION

The initial interaction between the male gamete and the female reproductive tract takes place at the uterine cervix. In order to achieve conception, normal shaped spermatozoa with progressive motility must be deposited at the cervical os in quantities sufficient to enable the sperm to overcome the barriers provided by the cervix, the uterine cavity, the oviducts and to reach the place of fertilization. As simple as this process may seem, abnormal interaction between spermatozoa and cervical mucus still remains one of the most difficult problems of infertility in particular in relation to an effective treatment.

The examination of postcoital cervical mucus for the presence of progressively motile spermatozoa in order to establish the receptivity in the periovulatory period to sperm penetration in the so-called post coital test (PCT) was first described by James Marion Sims in 1869 and popularized by the urologist Max Hühner in 1913 (1-3). Before that time, Donné (1844) was already calling attention to the acidity of the vaginal fluid in contrast to the alkalinity of the cervical mucus (4). The survival time of spermatozoa within the female genital tract has been the subject of great interest and investigation since then, in the beginning at the risk of offending professional morality and in the face of sharp criticism of colleagues (5, 6). Although the PCT is for more than half a century a basic part of any infertility evaluation, practitioners do not agree as to standard criteria for the interpretation of observations. There are many variables, such as the time in the cycle at which the test is performed, the latent period between coitus and examination of the cervical mucus, the temporal hiatus between coitus and preceding ejaculation, postcoital rest and posture as well as fractional testing (7).

Therefore it is extremely difficult to interpret the variety of results reported in the literature. There is also no real

standardization of definition as to what constitutes a normal post coital test, and many different methods have been advocated for classification of the results of the PCT. According to World Health Organization (WHO) criteria the test is considered to be normal when more than seven spermatozoa with good progressive movement and without signs of agglutination are observed per high power field (HPF = 400x). The PCT is inconclusive, when one to seven sperm cells with good motility are present in each field and the test is abnormal when either significant agglutination or immobilization of spermatozoa occurs or when complete lack of sperm cells in the mucus sample is encountered (8).

Since the spermatozoa only penetrate the mucus in the peri-ovulatory period for two or three days each cycle (9-13), adequate timing of the PCT is essential. Only during midcycle there is an increased secretion of mucus (up to 700 mg/day) with low viscosity (14). In 1972 Insler et al introduced a cervical score of 12 points in order to evaluate the physical properties of the cervical mucus (15). This is a simple semiquantitative method scoring by 0 to 3 points 1) the amount, 2) Spinnbarkeit and 3) crystallization capacity of the mucus and 4) the degree of opening of the external cervical os (table 1). The total score is graded in four categories: negative (0-3 points), initial (4-7 points), good (8-10 points) and excellent (11-12 points). An excellent correlation has been reported between the cervical score and the sperm penetrability (10). When considered with respect to the plasma luteinizing hormone surge (10, 16), the optimal cervix score and maximal linear sperm migration have been established on day -1 and 0, with a rapid decline on day +1, probably due to progesterone influence in combination with estrogen decline. The optimal cervix score in relation to the day of rupture of the dominant follicle has been found from day -3 onwards until day 0 (17).
Prediction of ovulation is possible by means of a semiquantitative late-morning urinary LH concentration. Data in literature about the moment of ovulation after the LH surge are inconclusive: they show a variation from approximately 4 to more than 40 hours after the LH surge (18-24). Besides that: the onset of the LH rise (LH

Table 1 Definition of scoring system

Parameter	0	1	2	3
Amounts of mucus	None	Scant: a small amount of mucus can be drawn from the cervical canal	Dribble: a glistening drop of mucus seen in the external os: mucus easily drawn	Cascade: abundant mucus pouring out out of the external os
Spinnbarkeit	None	Slight: uninterrupted mucus thread may be drawn approx. ¼ of the distance between the external os and vulva	Moderate: uninterrupted mucus thread may be drawn approx. ½ of the distance between the external os and vulva	Pronounced: uninterrupted mucus thread may be drawn for the whole distance between the external os and vulva
Ferning	None: amorphous mucus	Linear: fine linear ferning seen in a few spots; no side branching	Partial: good ferning with side branches in parts of the slide, linear ferning or amorphous mucus in other parts	Complete: full ferning of the whole preparation
Cervix	Closed: mucosa pale pink, the external os hardly admits a thin applicator		Partially open: mucosa pink, the cervical canal easily penetrable by an applicator	Gaping: mucosa hyperemic, the external os patulous

After Insler[15]: the cervical score

surge initiating rise (LH-SIR)) was found to predict far more accurately the ovulation time than the LH peak itself, the time of follicle rupture almost always being subsequent to the 37th hour after the LH-SIR (23-25). In order to rule out "pseudocervical hostility" (26-28) as the cause of a negative sperm penetration test (SPT), which just means inadequate timing, ultrasound examination has proven to be even more useful than urinary LH determinations (29). Not only a predictable relationship between follicle size and mucus quality (Insler score) has been established (17); it also has been demonstrated that ultrasound allows detection of ovulation in nearly all investigated cycles which renders this method an excellent tool for positive confirmation of ovulation (29). The advantage of ultrasound over other methods used for retrospective detection of ovulation (i.e. serum P, BBT) is that it can pinpoint the day of ovulation and not merely the fact that ovulation has occurred.

Furthermore: it seems that each patient has her own follicular development pattern (23). This means that after one inventory cycle it can be predicted for the following months at what follicular diameter the PCT, AIH or AID best can be performed.

When pseudocervical hostility is excluded, absence or immobilisation of sperm in the cervical canal may be due to unadmitted inability of intravaginal coïtus, ejaculatory problems (30), sperm abnormalities (31, 32), vaginal infection (33), ecto- and/or endocervicitis (34), sperm antibodies (35, 36), or mucus problems not related to the already mentioned causes (30, 32, 37, 38). The prevalence of infertility due to disturbed passage of spermatozoa in cervical mucus ranges in literature from 10-30% (32, 37-41).

The influence of pH and buffering capacity (BC)

Of the various causes which thus far have been implicated for incompatibility of spermatozoa with human cervical mucus, very little attention has been paid in recent literature to the influence of vaginal and cervical mucus pH upon the viability and motility of spermatozoa. The older literature data however clear-

ly pointed out that the hydrogen ion concentration is undoubtedly one of the most important factors that influence motility, viability and metabolism of spermatozoa in all species, including men (42-45). A pH just above 7 provides a normal situation for survival of spermatozoa whereas mild alkalinity has been observed to enhance the movement of spermatozoa. The motility of the spermatozoa however diminishes under strong alkaline conditions. On the other hand, the pH level of 6.0 has been established as the critical lower limit for the postcoital performance of spermatozoa (37, 46, 47). The lower the pH, the less the motility of the spermatozoa (e.g. immobilization within 30 seconds when pH < 4.7). Resuscitation by neutralization is, however, still possible, unless the pH has fallen below 3.5 (48).

Vaginal secretions are usually acidic, mainly due to lactic acid, with a pH varying from 3.5 to 5.1 (49-51). Cyclic vaginal pH variations have been described with decreased acidity at ovulation time (52-55) but these data have not been confirmed by others (50). The cervical mucus pH shows values ranging from 4.5 to 8.0. Literature data do not agree about the existence of a cyclic pattern of cervical mucus pH either (50, 56-58). The ectocervical mucus tends to have a lower pH as it comes in closer physical contact with the acidic vaginal fluid (7, 59).

Semen plasma consists predominantly of the secretions discharged by the accessory reproductive glands. The first fraction of the ejaculate is mainly prostatic fluid with a slightly acid pH (about 6.5), while the second fraction consists primarily of vesicular secretions (60). The pH of the whole ejaculate is usually stated to be in the range of 7.2 to 8.2 (42, 47, 61). Normally, the buffering capacity of the seminal plasma causes the vaginal pH to raise in a few seconds after ejaculation, thus preventing immobilization of the spermatozoa (50, 62, 63). This new pH level is maintained for several hours, which indicates the powerful buffering capacity of normal seminal fluid. Sexual arousal and its concomitant vaginal lubrication usually have little influence upon vaginal acidity: the buffering capacity of the transsudate is rather small (50, 62, 64). The film of seminal plasma in the

fornix posterior also forces a pH rise upon the cervical mucus, which favours the penetration of spermatozoa (50). The optimal pH for sperm migration and survival in cervical mucus ranges between 7.0 and 8.5 (65, 66). In cases of repeated negative PCT's because of an abnormal pH of the lower genital tract (37, 67) or because of diminished buffering capacity of the ejaculate (50) vaginal irrigation with sodium bicarbonate preceding intercourse has been advocated in literature.

Only few data on the buffering capacity of animal semen and even fewer on human semen have been reported (68-72). Investigation of bull semen has established that the buffering capacity was higher in the acid (pH 4-5.5) than in the alkaline region (pH 9-10) (69, 72). In the pH range between 5.5 and 9 the BC proved to be relatively poor. For the human ejaculate also a higher buffering capacity has been found in the lower pH area (73). Furthermore, in bull semen a significant negative correlation has been assessed between the BC and the pH, but a significant positive correlation between the BC and the motility of spermatozoa. No significant correlation has been found between the buffering capacity, the volume of the ejaculate and the sperm count (per ml) respectively (72, 74). The buffering capacity has been attributed primarily to citrate, phosphate and bicarbonate (42, 69, 72). The titration curves of bull seminal vesicle fluid are remarkably similar to the curves of complete semen samples, which suggests that seminal vesicle fluid contributes much to the BC of semen (42, 69). This item however has not been further investigated. Another interesting experience has been, that the BC of bull semen diminishes with storage (69). Data reported about human semen are not consistent upon this item. One report suggests that semen becomes more alkaline on standing, most likely due to loss of carbon dioxide (71). Later on the pH of the semen showed the tendency to decline which change has been attributed to the formation of lactic acid and other acids (71). Another publication reports no rise of pH on standing at all, but an immediate decline of pH with a further enhancement of buffering capacity subsequently (73). In comparison with serum, bovine semen seemed to have a

higher BC, except in the limited range near neutrality (69). The observations in these reports should be viewed as preliminary and incomplete ones. This led us to the present study reported in this thesis.

Van Slyke has described a way for experimental determination of the buffering value of a solution, which means the ability of that solution to resist change in pH through the addition or loss of alkali or acid (75). The unit adopted for the buffering effect has been the differential ratio dB/dpH, expressing the relationship between the increment of stong base (dB) added to a buffer solution and the resultant increment in pH (dpH). If acid has been added both dB and dpH are negative, so the ratio is always a positive numerical value. This unit of buffering capacity has been called slyke since.

The influence of viscoelasticity of cervical mucus

Cervical mucus is a hydrogel, that shows a cyclic variation in viscoelasticity and consists of two fractions with rheologic different properties (76, 77). The high-viscosity component (HVC) exists of water insoluble glycoproteins (= mucins) that are responsible for the gel formation (77). The amount of HVC as percent of total declines in the preovulatory period from 7% to 1%. The low-viscosity component (LVC) or cervical plasma is composed of water with water soluble proteins, salts and other components with low molecular weight. The amount of LVC enhances in the preovulatory period through stimulation of the cervix by estrogen to 99%, thus impeding the viscoelasticity of the cervical mucus. Malfunction of this hormonal regulating mechanism (mainly follicular dysfunction) or inadequate response of the endocervical epithelium (viz. narrow mucus window or hyporesponsive cervix (17)) may result in significantly impeded sperm transport and infertility due to the so-called "cervical factor" or dysmucorrhea (41). The penetrability of cervical mucus will however be increased when exposed to a concentration of an inorganic salt containing preferentially multivalent anions at a concentration

in excess of 0.05% (wt/vol) (67). This observation has created a potential new indication area for vaginal irrigation with sodium bicarbonate prior to sexual intercourse, viz. periovulatory mucus with high viscoelasticity. As mentioned before, previously irrigation with an alkaline solution (e.g. $NaHCO_3$) was only prescribed to improve vaginal and endocervical mucus pH in case of repeated negative PCT's due to acidity (37, 50, 67).

Aim of this study

The buffering capacity of semen is a defense mechanism against endogenous and exogenous influences upon semen pH, creating an environment favouring the penetration of spermatozoa in the cervical mucus. Since acidity seems to be detrimental to sperm motility we hypothesized that the buffering capacity might be deficient in some cases and therefore shortage of BC might be one of the causes of infertility. In literature e.g. low seminal plasma volume ($\leq$ 1.5 ml) is equated with poor buffering capacity (62).

Human semen samples of all fertility grades (78) were incorporated in our study in order to determine the buffering capacity from titration curves (chapter II). Following Woodbury (79) and Izutsu (80) the BC was defined as the number of micromoles HCl that has to be added to 1 ml semen to lower the pH from 7 to 6, with the Slyke as unit of BC (75). The steeper the slope of the titration curve, the more the solution is resisting a change of pH, which means: the higher the buffering capacity. We aimed at establishing (1) the variability in semen BC between individuals and with time, (2) a possible relationship between BC and fertility grade and (3) the pH range with the maximal BC. Furthermore, we wondered what position the buffering capacity of human semen would hold in the row of BC's of other body fluids. Therefore a comparison has been made between the BC of semen and serum of the same man (own observations), saliva (80) and tear fluid (81) (literature data). Also a comparison has been made between human

and animal semen buffering capacity (72-74).

In the treatment of some causes of infertility arteficial insemination (AIH) with the first fraction of a split ejaculate is advocated (82, 83). Indication criteria for the splitting procedure are deficiences in sperm count (per ml) or sperm motility and increased semen viscosity (83). There are at least two arguments in favour of this policy: the first fraction contains a significantly higher sperm count and the spermatozoa have a better progressive motility in comparison with the whole ejaculate (82). An essential condition for this kind of treatment is however a relatively large ejaculate volume. We wondered whether the advantages of the first fraction of the split ejaculate might be partially counteracted by potential lack of sufficient BC (chapter III). Therefore the BC of the two split ejaculate fractions has been determined and compared with the BC of the whole ejaculate.

Little is known about the buffering substances of semen: it has been suggested that the BC of semen depends mainly upon citrate, bicarbonate and other low molecular substances (69, 72). We have investigated the contribution of the HCO_3^-/CO_2 system, high molecular weight components (proteins) and spermatozoa to the buffering capacity of semen (chapter IV).

Vaginal irrigation with an alkaline solution, as mentioned before, has been advocated in literature as a therapy in cases of infertility caused by excessive acidity in the lower genital tract or periovulatory mucus with high viscoelasticity (37, 50, 62, 67). The composition of the irrigation fluid varies in literature from the use of a pH 7.4 buffer (62) to a mixture of 1 tablespoonful of $NaHCO_3$ in 1 quart of tap water (67) or 30 g $NaHCO_3$ per liter (50). The latter $NaHCO_3$ compositions result in solutions with a sodium bicarbonate concentration of 1.4% and 3% (wt/vol), respectively. It is generally accepted that spermatozoa are sensitive to variations in osmotic pressure and cannot survive under substantial changes in osmolality (43, 47). We have investigated the effects of hyper- and hypoosmolality upon the viability and

motility of spermatozoa (chapter V). We have used the 1.5% (wt/vol) sodium bicarbonate solution in the subsequent experiments, because the osmolality of this solution proved to be identical with the slight hyperosmotic values of semen samples shortly after ejaculation. In an in-vitro study we have tried to confirm the beneficial effect of $NaHCO_3$ douching upon the indications described in literature (50, 62, 67). We therefore have examined the SPT-score and viscoelasticity of cervical mucus incubated with 1.5% (wt/vol) $NaHCO_3$ as compared to control mucus. In order to prove the effect of sodium bicarbonate irrigation a double-blind prospective randomized clinical trial has been performed (chapter VI). The viscoelasticity of periovulatory cervical mucus and sperm penetration in vitro (SPT) and in vivo (PCT) have been compared in three situations: (1) after vaginal irrigation with 1.5% (wt/vol) $NaHCO_3$ or (2) after irrigation with 0.9% (wt/vol) NaCl versus (3) the control cycle in which no irrigation has been performed.

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CHAPTER II

BUFFERING CAPACITY OF HUMAN SEMEN

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ABSTRACT

The buffering capacity of 270 semen samples derived from 196 men of infertile couples was determined from titration curves. The average buffering capacity in the physiological range (pH 7.0 - pH 6.0) was 41.1 slyke (standard deviation [SD] ,9.9), which is significantly higher ($P < 0.01$) than that in serum (23.3 slyke; SD, 7.5, n = 42). When the buffering capacity of several semen samples of one man in the course of time was measured, the variation between these samples was larger than the determination error. No correlation was found between the buffering capacity of serum and semen of the same man, nor between the buffering capacity of semen and the fertility grade determined by physical and morphological analysis of the samples.

INTRODUCTION

The buffering capacity (BC) of semen is a defense mechanism not only against endogenous influences on the pH but also against exogenous influences. The process of fructolysis is the endoge-

nous factor most likely to lower the semen pH below the optimal value. This process yields the energy for the spermatozoa and results in lactic acid accumulation, leading to a decrease in the semen pH. The acid pH normally existing in the vagina is the exogenous influence on the semen pH. If not compensated by the BC of the semen, the resulting low pH will lead to immobilization and eventually death of the spermatozoa[1,2]. However, normally the originally low pH in the vagina is raised within a few seconds after ejaculation of semen, which pH level is maintained for several hours, with only a gradual pH decline[3]. Thus the buffering capacity of semen creates an environment favouring the penetration of spermatozoa through the cervical mucus. One of the causes of infertility might thus be a low BC of the semen. In that case a vaginal washing with an alkaline solution prior to intercourse could be of therapeutic value by maintaining a sufficiently high vaginal pH value to allow protection of the spermatozoa[3].

Only few data on the BC of animal semen and even fewer on human semen have been reported[4-8]. There is agreement among several investigators that bull semen is more highly buffered on the acid than on the alkaline side. Anderson[8] describes the BC of bull semen as the change in pH after addition of 0.05 ml of 0.1 N HCl to 0.15 ml seminal plasma, which amounts to the following value: 1.84 ± 0.39. Srivastava[9], applying the same method, calculates for the BC of buffalo semen a value of 1.40 ± 0.18. Anderson[8] finds a significant, positive relationship between the motility of bull spermatozoa after 24 hours and the BC of bull semen, but Srivastava[9] cannot confirm this for buffalo semen.

No reliable values for the BC of human semen have been reported, and a relationship with the fertility grade of the semen[10] has not been described. Therefore, we have studied the BC of semen from a large number of men in order to investigate its possible relationship to the fertility grade and to determine variability between individuals and with time.

DEFINITION OF BUFFERING CAPACITY

Biogical fluids are usually characterised by a rather constant pH value, which is not greatly altered by the addition of acid or alkali. This property can be quantitatively expressed in the BC. The BC is defined as the slope of the titration curve when the fluid is titrated with strong acid or base, in mathematic form:

$$BC = \frac{1}{\Delta pH} \times \text{equivalent added acid or alkali per milliliter fluid}$$

The BC thus depends on the pH of the fluid, and hence it is necessary to define a certain pH range for which the BC is determined. Plots of pH changes against amounts of acid added showed that the slope of the titration curve for human semen is rather constant between pH 6 and 7. This is a physiologically interesting range, because spermatozoa begin to be immobilized when the semen pH falls below 6[11].

Following Woodbury[12] and Izutsu[13] we used the slyke as the unit of buffering capacity, defined as the number of micromoles HCl that must be added to 1 ml semen to lower the pH from 7 to 6.

MEASUREMENT OF BUFFERING CAPACITY

Semen samples are collected in closed amber glass funnels and stored after 3 hours' prolonged standing in closed glass test tubes at -20°C. When the BC is to be measured, the sample is thawed and vortexed. Then a 0.2 ml aliquot is transferred to a plastic sampling vessel, containing 2 ml 0.9% (wt/vol) NaCl (no differences in BC values are observed in several dilutions, between a range of 0.05 and 0.8 ml aliquot). A drop of octanol is added to prevent splashing during titration. The samples are

then titrated with 0.1 M HCl at 20°C in a recording pH meter (RTS 822, Radiometer, Copenhagen, Denmark). After reading the initial pH, automatic titration is performed in the pH range from 8.0 to 5.2 (Fig. 1) at an initial titration speed of 0.3 ml/min, and gradually reduced upon approaching the end point of 5.2 within 0.05 pH units. A single titration run takes approximately 4 minutes; triplicate runs are made for each sample. From these titration curves the BC can be calculated.

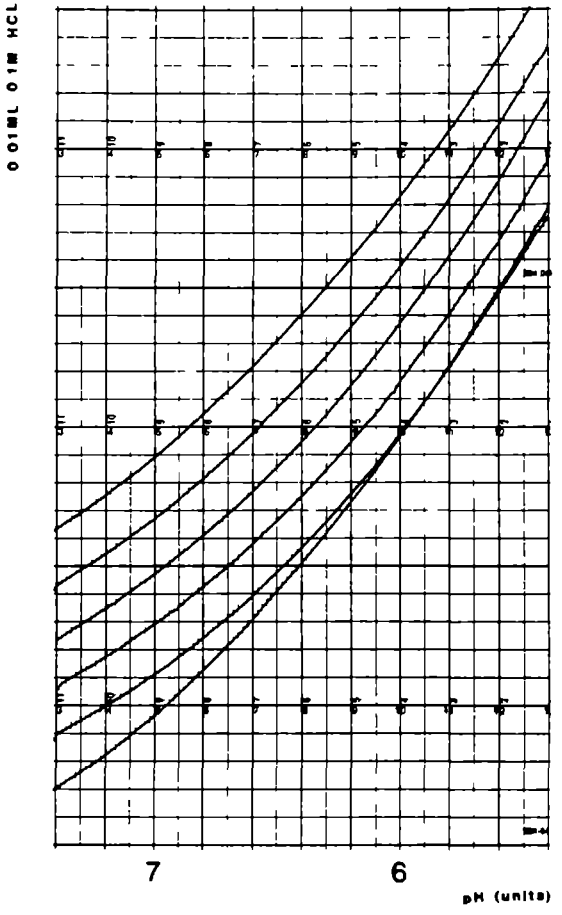

Figure 1

Titration curves for a single semen sample. Reduced in size (0.01 ml = 0.5 cm).

CHARACTERIZATION OF THE SEMEN SAMPLES

A total of 270 semen samples, obtained from the male partners of 193 infertile couples, were investigated. The mean number of days the couples abstained from sexual intercourse was 4.5 (standard deviation [SD] = 3.1; n = 137). No correlation was found by means of the Kendall correlation test, between the abstinence period and the pH value ($P = 0.93$), or between the abstinence period and the BC ($P = 0.32$). The possible influence of the interval between obtaining samples and analyzing them and of the freezing procedure upon the pH and buffering capacity has been excluded in the next experiment. Semen samples of 6 individuals have been analyzed within 15 minutes after ejaculation, after 3 hours' prolonged standing at room temperature and after 12 days' storage at -20°C. Table 1 shows that there was significant difference neither in initial pH nor in buffering capacity of these samples. Therefore, further samples were frozen within 3 hours after ejaculation, in which period routine analysis was performed.

For statistical reasons only the first samples of the 193 men were used for establishment of the distribution of semen characteristics and for the investigation of the relation between BC and fertility grade. Repeated samples obtained from a number of men over the course of time were used to elucidate the variability of the BC for individuals.

The semen characteristics, which have been examined, are summarized in Table 2. The volumes were directly read in 0.1-ml units from the scale division upon the collecting funnel. pH indicator papers were used for pH determination. The viscosity was judged by pulling threads: up to 1 cm is normal; < 1 cm is abnormal. Sperm motility after incubation for several minutes at 37°C is expressed on a scale of 0 (immobile) to 6 (maximal motility). The number of living spermatozoa and their morphologic features were determined in a methyl blue/eosin preparation (dead cells staining red). The living cells were screened for morphological abnormalities. The number of spermatozoa was deter-

Table 1. Effect of Prolonged Standing and Storage at -20°C upon pH and BC of Semen Samples[a]

	pH			BC		
	Fresh (within 15 min)	After 3 h. standing	After storage at -20°C	Fresh (within 15 min)	After 3 h. standing	After storage at -20°C
1	7.46 ± 0.05	7.49 ± 0.01	7.50 ± 0.0	33.5 ± 0.5	35.7 ± 1.5	33.3 ± 0.8
2	7.93 ± 0.02	7.96 ± 0.01	7.95 ± 0.01	32.3 ± 0.8	32.0 ± 0.2	31.0 ± 0.8
3	7.87 ± 0.03	7.84 ± 0.01	7.84 ± 0.01	43.0 ± 0.5	41.0 ± 1.5	42.5 ± 0.5
4	7.95 ± 0.02	7.99 ± 0.03	8.01 ± 0.03	43.5 ± 1.5	40.5 ± 0.5	41.0 ± 0.5
5	7.69 ± 0.01	7.66 ± 0.01	7.69 ± 0.01	33.8 ± 1.8	35.5 ± 0.5	34.0 ± 1.5
6	7.58 ± 0.03	7.58 ± 0.03	7.63 ± 0.03	34.0 ± 0.5	32.5 ± 1.5	32.5 ± 0.5

[a] the data are mean values ± SD of three determinations of each sample

Table 2. Semen Characteristics of the Samples Gathered from Men of Infertile Couples (n = 193)

	Mean	SD	Range	n	normal	percentage in normal range
volume (ml)	3.85	2.10	0.4- 13	193	2- 5	61.7
viscosity: 1 = normal						
2 = abnormal				193	1	84.1
pH	7.48	0.23	7.1-8.9	193	7- 8	96.9
number of spermatozoa(x 10^6/ml)	53.8	54.9	0-285	193	20-200	63.2
living spermatozoa (%)	79	17	0-100	182	> 60%	92.3
motility grade[a]	4.1	1.3	0- 6	188	4- 6	68.1
motile spermatozoa (%)	74	36	0-100	186	> 50%	74.2
teratozoospermia (%)	47	16	20- 94	178	< 40%	41.6
heads	42	17	5- 93	178		
mids	3	2	0- 13	178		
tails	2	3	0- 20	178		
fructose content (mmol/L)	14.4	6.7	0.7-25	184	> 7	85.9
fertility grade	11.6	4.1	0- 16	193	> 11	67.3

[a] 1 = no movements; 2 = bad; 3 = reasonable; 4 = rather good; 5 = good; and 6 = excellent

mined in a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). The fructose concentration was determined by the hexokinase/glucose-6-P-dehydrogenase method, with and without conversion of fructose-6-P to glucose-6-P by the enzyme phosphoglucose isomerase[14]. The fertility grade of the samples was calculated with the aid of a points system, according to Kremer[10] (Table 3 and 4).

RESULTS

Of the 193 first or only semen samples the pH value and BC were determined simultaneously. The distribution of the pH and of the BC values is given in Figure 2A and 2B. The mean pH

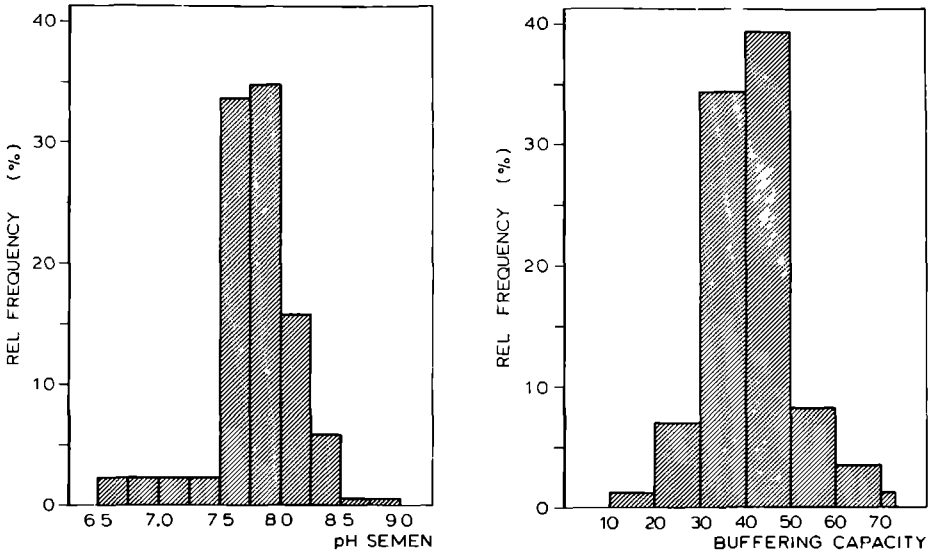

Figure 2

(A), Histogram for pH of semen samples. (B), Histogram for BC of semen samples.

Table 3. Evaluation of the Ejaculates in Terms of the Fertility Grade According to Kremer[10]

Fertility grade		n	%
sterile	= 0 points	7	3.6
bad	= 1- 6 "	12	6.2
poor	= 6-11 "	45	23.3
passable	= 11-13 "	31	16.1
good	= 13-16 "	98	50.8
Total		193	100.0

Table 4. Fertility Grade According to Kremer

	Points[a]				
	0	1	2	3	4
count in millions/ml	0	< 5	5-20	20-60	> 60
percentage motile	0	< 5	5-40	40-60	> 60
grade of motility	< 1	1- 2	2- 3	3	4- 5
morphology (% normal heads)	< 20	20-50	50-60	60-70	> 70

[a] The points obtained with these four parameters are added, and the sums are used for assigning the fertility grades.

value is 7.80, and the mean buffering capacity is 41.1 slyke with an SD of 9.9 (Table 5). When the samples are ranged according to their fertility grade, the BC is 37.5 slyke in the sterile group (SD = 6.2), 42.9 slyke in the bad (SD = 12.0), 43.7 slyke in the poor (SD = 9.8), 40.2 slyke in the passable (SD = 8.8) and 40.4 slyke in the good samples (SD = 10.1). The distribution of the BC between the several groups does not differ significantly (Kruskal-Wallis test, P = 0.19). There is a considerable variation among the samples within each group, for which no explanation could be found, e.g., the various times of previous ejaculation.

Table 5. pH and BC Values of the Semen Samples

	n	Mean	SD	Range
pH	193	7.80	0.28	6.44- 8.64
BC (slyke)	193	41.1	9.9	12.0 - 72.5
total BC (slyke.ml)[a]	193	161.0	107	12.2 - 661.3

[a] total BC = volume x BC

For 19 men the BC of three or more sequential semen samples was determined. The BC's of these samples are given in Figure 3. It is clear from this illustration that the BC of semen varies among men. Preliminary statistical analysis of variance performed on a number of triplicate measurements of the same semen sample shows that the relative error in the determination of the BC is 5%. The relative SD of the BC for the samples obtained at different times from the same man exceeds this 5% in more than 50% of the cases, indicating that the BC of the semen of one man

varies in time. Therefore, several semen samples of one man should be measured for one to obtain a characteristic value for the BC of this person.

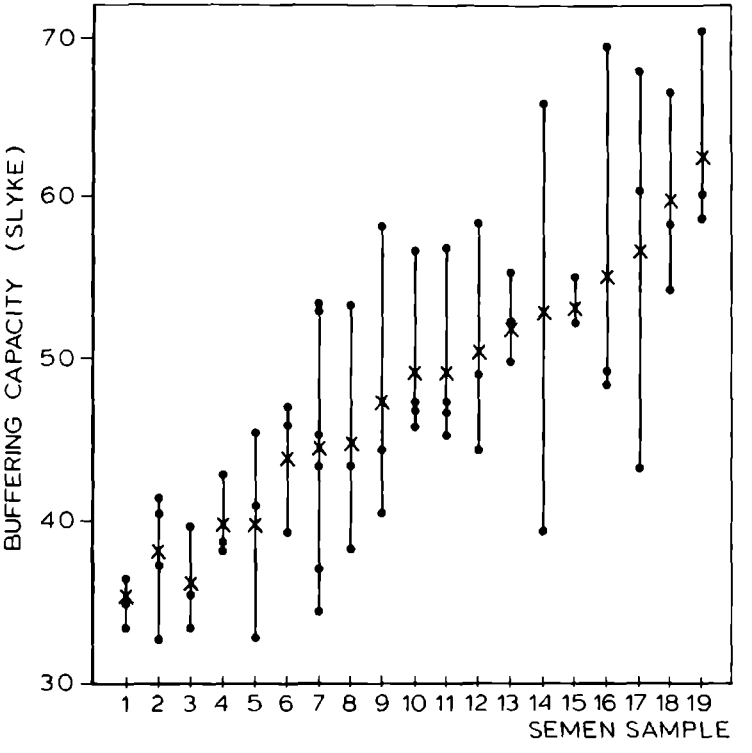

Figure 3

BC's determined in three to six samples obtained at different times from 19 subjects. Individual values are represented by dots; the means, by crosses.

For 42 men the BC of the serum was also measured. The mean serum BC is 23.8 slyke (SD, 7.5), which is 0.51 times that of the average BC of semen. The values of the serum BC were compared with those of the semen BC for the same man, for determination of whether the two values correlated (Fig. 4). No correlation could

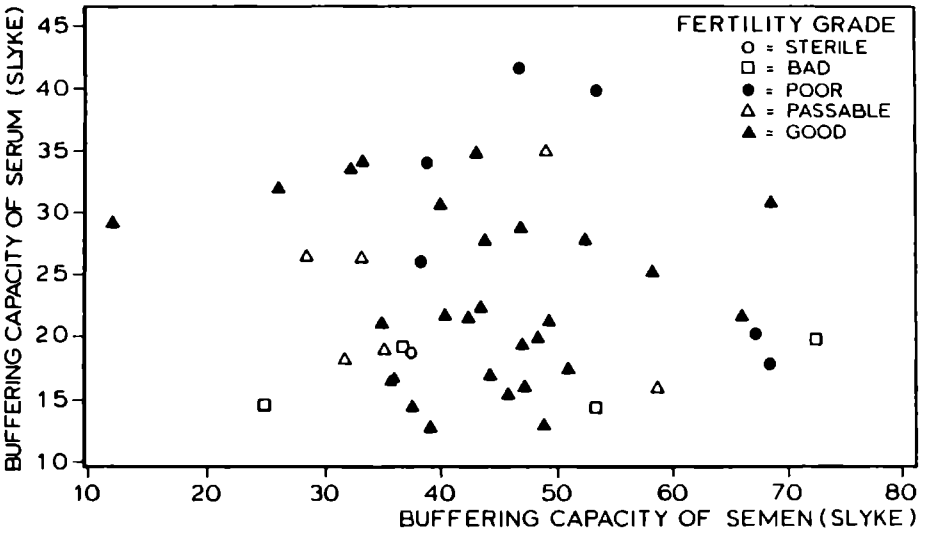

Figure 4

Serum BC values plotted against the paired semen BC values for 42 subjects. Fertility grade is indicated by symbols, as shown in the inset.

be found between the two parameters by means of the Kendall correlation test (P = 0.63).

From the complete titration curve, the determination of the pH range with the maximal BC is possible. In accordance with the findings of Bartek[15], the maximal BC value (44.9 slyke) is found in the pH range of 6.0 to 6.5, the BC in the pH range of 5.5 to 6.0 being only slightly lower (43.5 slyke). Above pH 6.5 the BC declines more rapidly: 35.9 slyke in the pH range of 6.5 to 7.0 and 24.7 slyke in the pH area of 7.0 to 7.5.

DISCUSSION

The results presented in this paper indicate that it is unlikely that the BC of human semen perse is a major determining factor in male infertility.

There is no correlation between the BC and the fertility grade determined from morphologic, physical and biochemical criteria. Because the total BC of semen is the product of BC and volume, however, one of the reasons for infertility might be low total semen BC.

The BC of human semen appears to be higher than that of bull and buffalo semen, reported by Anderson[8] and Srivastava[9]. Their values are not directly comparable with ours because of the difference in definition of the BC. They describe the BC as the change in pH after addition of 0.05 ml of 0.1 N HCl to 0.15 ml seminal plasma, whereas we use the slyke as unit of BC: the number of micromoles HCl that has to be added to one ml semen to lower the pH from 7 to 6. From our titration curves, we can calculate that with their procedure human semen would give a pH change of only 0.8 units, which is much less than their values for bull or buffalo semen (1.84[8] and 1.40[9]). This means that the BC of human semen is much higher than that of bull and buffalo semen.

The BC of human semen is also much higher than that of other human body fluids. We find a mean BC of semen of 41.9 slyke,

whereas the mean BC of serum is only 23.3 slyke. The BC reported for saliva is even lower, namely 14.2 slyke[13]. The BC of human tear fluid, calculated from the data of Carney[16], is only about 7% of that for human semen.

Although our 270 semen samples were all obtained from the male partners of infertile couples, this does not mean that these samples all had bad fertility grades. Half of them have a good and nearly 20% a passable fertility grade, levels which should enable them to bring about pregnancy. Actually, there is a good spread of our samples over the entire range of fertility grades. This has allowed us to test for correlation between fertility grade and semen BC. No such correlation appears to exist in our experimental material. This indicates that the semen BC cannot be an overruling determinant of fertility.

The mean pH value recorded upon measuring the BC is 0.32 pH units higher than the mean value found with routine laboratory examination immediately after collection. The techniques used in these two procedures are different: pH indicator paper in the routine test, the electrometric method in the BC determination. However, the consistently higher pH value in the later determination suggests that the difference is at least partly due to loss of some CO_2 during the period between ejaculation and analysis, resulting in a rise in pH and a fall in BC. This would imply that our BC values represent a lower limit. The total volume of the ejaculate and its sperm density in conjunction with the pH of the cervical mucus may be the more important factors in determining fertility.

Nevertheless, an investigation of whether in cases of low BC of the semen an alkaline vaginal washing prior to intercourse might be a possible therapeutic procedure would stille seem worthwile.

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CHAPTER III

BUFFERING CAPACITY OF FIRST AND SECOND FRACTIONS OF SPLIT EJACULATES

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ABSTRACT

The buffering capacity of first and second fraction of split ejaculates of 27 semen samples derived from 11 men of known infertility was determined from titration curves. The average buffering capacity of the first fraction in the physiological range (pH 7.0 to 6.0) was 33.1 $\pm$ 6.7 which was significantly lower ($P < 0.01$) than the average buffering capacity of the second fraction (44.6 $\pm$ 10.8 slyke). No correlation was found between the buffering capacity of the different semen fractions and the fertility grade, determined by physical and morphological analysis of the split samples.

INTRODUCTION

The buffering capacity (BC) of semen is in principle an important factor in the process leading to pregnancy[1]. Recently we studied the BC in 270 semen samples derived from 196 men of known infertility[2]. Results in this study indicated that the BC of semen was high as compared to that of other body fluids and that there was no correlation between the BC of semen and the fertility grade determined on the basis of different criteria.

Use of the first fraction of a split ejaculate for artificial insemination (AI) is advocated in the treatment of some forms of male and female subfertility. There are at least two arguments in favour of this policy. The first fraction of a split ejaculate contains most of the spermatozoa which have higher sperm motility[3,4]. It is not clear however, whether these advantages were partially counteracted by the lack of sufficient BC in the first ejaculate fraction.

Therefore the objective of the present study was to determine the BC of the two split ejaculate fractions, and compare them with the BC of the whole semen sample. Furthermore, to draw any possible correlations between the documented BC and the fertility grade of the two fractions of the split ejaculate.

MATERIALS AND METHODS

DEFINITION OF BUFFERING CAPACITY

The BC is defined as the slope of the titration curve when the fluid is titrated with strong acid or base. The slope of the titration curve for human semen is rather constant between pH 6 and 7, the physiologically important range. Following Woodbury[5] and Izutsu[6] we used the slyke as the unit of BC, defined as the number of micromoles of HCl that must be added to 1 ml semen to lower the pH from 7 to 6.

MEASUREMENT OF BUFFERING CAPACITY

The method of measuring the BC has been extensively described in a previous study[2]. Briefly each sample consisted of 0.2 ml semen; two ml 0.9% (wt/vol) NaCl and a drop of octanol were added to the semen sample to prevent splashing during titration. Automatic titration was performed using a recording pH meter (RTS 822, Radiometer, Copenhagen, Denmark). Triplicate runs were performed for each sample and the BC was calculated from these titration curves.

ASSESSMENT OF THE SEMEN SAMPLES

Twenty seven semen samples from 11 men of known infertility were examined. To collect the split ejaculate specimen, the patients were provided with two wide-mouthed jars. They were told to get the first few spurts of the ejaculate into the first jar and the remainder in the second one. Ideally, the specimen was divided into one-third of the volume in the first fraction and the latter two-thirds in the second fraction. Not everyone has been successful in this manoeuvre which may be evident from the volume ratio: ideally the quotient of second and first fraction equals 2, but in only 40% of the samples this quotient lies between 1 and 3, and in not more than 65% this quotient is larger than 1.

In a previous study[2] no correlation was found between the sexual abstinence period and the BC. Also, neither the time interval between the time of obtaining the samples and the semen analysis, nor the freezing procedure had any effect upon the pH and BC. The samples were frozen within 3 hours after ejaculation, in which period routine analysis was performed. The laboratory procedures have been previously described[2]. The semen parameters measured are summarized in Table 1.

The fertility grade of the samples, given in Table 2, has been calculated with the aid of a points system, according to Kremer[7].

Statistical analysis of the data was performed by means of a sign test.

RESULTS

Statistical comparison by performing a sign test between both fractions has shown that the pH distribution was about the same for both fractions, while the fructose concentration was higher in the second fraction. As was expected the total number of spermatozoa in the first fraction is higher. The fertility grade of the first fraction was also higher than that of the second fraction (Table 2).

TABLE 1. Semen Characteristics of the First and Second Fractions of ejaculates used in present study.

	FIRST FRACTIONS			SECOND FRACTIONS			
	Mean ± SD	Range	n	Mean ± SD	Range	n	P[2]
Volume (ml)	2.31 ± 1.53	1.0 - 7.0	27	3.91 ± 2.27	0.30-9.00	27	-
Viscosity: 1 = normal	23 = 1		27	25 = 1		27	-
2 = abnormal	4 = 2			2 = 2			-
pH	7.58 ± 0.27	7.20- 8.40	27	7.63 ± 0.32	7.30-9.00	27	-
No. of spermatozoa (x 10^6/ml)	32.9 ± 43.4	20 - 200	27	4.6 ± 5.5	0.5 -24.0	27	0.04
Living spermatozoa (%)	81.0 ± 10.0	60 - 94	27	75 ± 21.0	0 - 94	27	-
Motility grade[1]	3.8 ± 1.2	1 - 5	27	3.2 ± 1.1	1 - 5	27	-
Motile spermatozoa (%)	75.0 ± 35.0	0 - 100	27	66 ± 43.0	0 - 100	27	-
Fructose content (mmol/l)	10.4 ± 4.7	1.4-18.9	22	16.7 ± 6.4	3.5-28.6	23	0.07
Fertility grade	11.1 ± 2.6	3 - 16	27	9.2 ± 2.7	1 - 13	27	0.04

[1] 1 = no movements; 2 = bad; 3 = reasonable; 4 = rather good; 5 = good; and 6 = excellent

[2] P value between first and second fractions determined by means of a sign test

TABLE 2. Evaluation of the Ejaculates in Terms of the Fertility Grade[1]

	FIRST FRACTIONS		SECOND FRACTIONS	
Fertility Grade	n	%	n	%
Sterile = 0 points	0	0	0	0
Bad = 1- 6 "	1	3.7	2	7.4
Poor = 6-11 "	8	29.7	16	59.3
Passable = 11-13 "	13	48.1	7	25.9
Good = 13-16 "	5	18.5	2	7.4
Total	27	100	27	100

[1]Fertility Grade accordering to Kremer[7]

The pH value and BC of the first and second fraction of 27 semen samples were determined simultaneously. The distribution of the pH and of the BC values for both fractions is shown in Figures 1 and 2.

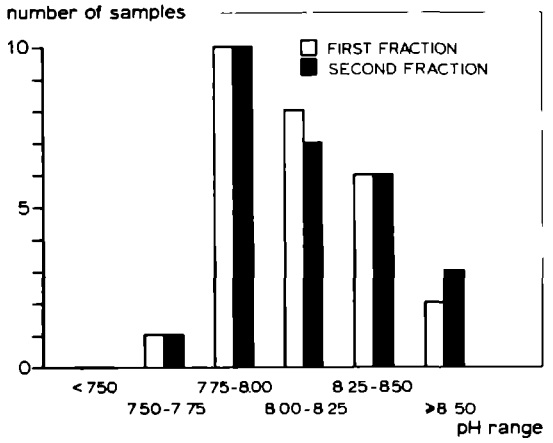

FIGURE 1. The pH of the two ejaculate fractions obtained via split ejaculation

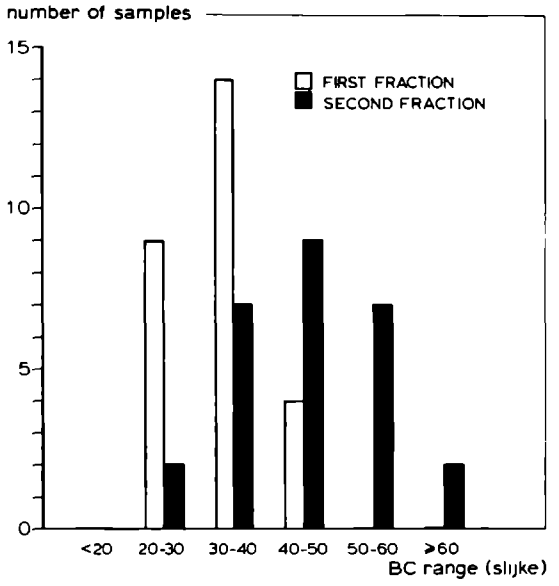

FIGURE 2. The buffering capacity of the two ejaculate fractions obtained via split ejaculation

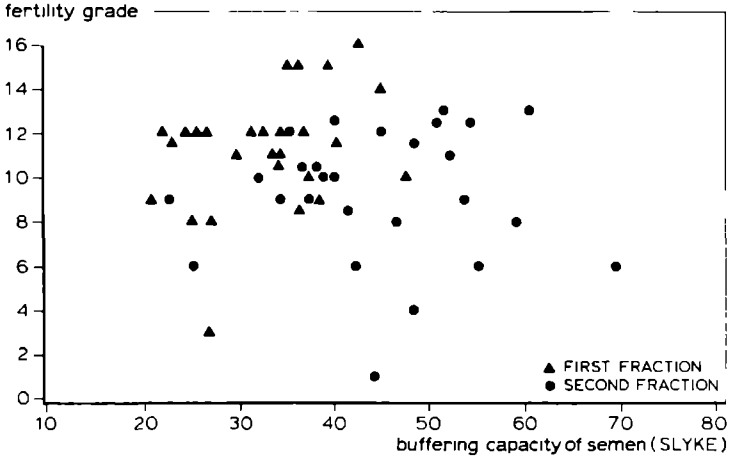

FIGURE 3. Fertility grade of first and second fractions of the split ejaculate plotted against semen buffering capacity

The mean pH value is about the same for the first and second fraction (8.14 and 8.13, respectively; Table 3). The mean (± SD) BC of the first and second fraction were 33.1 ± 6.7 and 44.6 ± 10.8 slyke respectively. This difference was statistically significant (P = 0.01). No correlation was found by means of the Kendall correlation test, between the fertility grade and the BC of the first fraction (P = 0.42), or the second fraction (P = 0.59; Figure 3).

DISCUSSION

The spermatozoa showed higher concentrations, motility and vitality (motility grade) in the first fraction than in the second fraction of the split ejaculate. The fertility grade of the first fraction was calculated to be 2.2 points higher than that of the second fraction. These results are in agreement with previously generated data[4,8]. The depressed sperm motility and vitality noted in the second portion of fractionated ejaculates might be attributed to a suppressive factor in the seminal vesicular fluid deleterious to human sperm survival[4] or to a higher concentration of a metabolic regulator[8] which in some manner uncouples oxidative phosphorylation, decreasing in turn the respiratory inhibition of glycolysis. However the mean BC of the first fraction was significantly lower ($p < 0.05$) than that of the second fraction. It is these authors' opinion that an ideal ejaculate splitting condition, resulting in a volume ratio of 1 : 2, could make this difference even greater.

Whether the relatively low BC of the first fraction is capable to accomplish sufficient elevation of the vaginal pH, it has to be questioned. This will depend mainly upon the volume of the first fraction. Without being exceedingly specific it can be reasoned that the first ejaculate fraction has to meet a certain critical volume to overcome the acidic pH of the vaginal cavity. So, under these circumstances, an essential splitting condition to meet the necessary requirements would necessitate a relatively

TABLE 3. The pH and Buffering Capacity (BC) values of the two ejaculate fractions obtained via split ejaculation

	FIRST FRACTIONS			SECOND FRACTIONS			
	n	Mean ± SD	Range	n	Mean ± SD	Range	P**
pH	27	8.14 ± 0.27	7.74- 8.65	27	8.13 ± 0.32	7.74- 9.20	0.54
BC (slyke)	27	33.1 ± 6.7	20.7 - 47.6	27	44.6 ± 10.8	22.5 - 69.3	0.01
Total BC*	27	77.0 ± 47.0	27.0 -240.0	27	181 ± 100	7.6 -360	

* Total BC = ejaculate volume x BC (slyke/ml)

** P value between first and second fractions determined by means of a sign test

large semen volume. In cases that this condition cannot be met, the confrontation with the acid environment of the vagina can be avoided by either cervical cap insemination of the first fraction or vaginal alkaline douching preceding insemination in the cervical mucus and fornix posterior. When these methods do not succeed only intrauterine insemination using washed spermatozoa is left[9].

As in the previous study[2] the pH values of the semen samples were higher (± 0.5 pH unit) than those determined in the routine laboratory. The reason for it has been discussed before[2]. However, the pH value of the two fractions is hardly different in each of the determinations, which is in contrast with literature data[3,10].

The theoretical total buffering capacity of the combined fractions, which was calculated from the volumes and BC of the two separate fractions was 41.8 slyke. This value is compatible with the mean (40.2 slyke) of the 193 examined complete ejaculate samples, described previously[2]. About 15% of these complete samples had a BC below 33.1 slyke, which was the average BC of the first fraction. In the previous study[2] no relationship was found between low BC and the fertility grade. This finding suggests that the lower BC in the first fraction as compared to the total ejaculate is not a serious drawback against the use of this fraction in treating some forms of male subfertility.

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CHAPTER IV

BUFFERING SUBSTANCES OF HUMAN SEMEN

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ABSTRACT

The quantitative contribution of several components to the buffering capacity (BC) of human semen has been investigated. The role of spermatozoa is negligable (less than 2%). Both the high-molecular components (proteins) and the HCO_3^-/CO_2 system contribute about 25% to the BC. Therefore about 50% of the BC of semen must be due to low molecular weight components other than HCO_3^-/CO_2.

INTRODUCTION

In a previous study[1] the BC of a large number of human semen samples was determined. This BC turns out to be high when compared to other human fluids, such as blood serum. Although the composition of semen of different species is known in detail from the work of Mann,[2] little is known about the buffering substances of semen. It has been suggested that the BC depends mainly upon citrate, bicarbonate and other low molecular substances.[2,3] We investigated which contribution the HCO_3^-/CO_2 system and high molecular components (proteins) offer to the BC of semen.

The method of measuring the BC has been described previously.[1] In summary: each sample consisted of 0.2 ml semen; 2 ml 0.9% (wt/vol) NaCl was added, and a drop of octanol to prevent splashing during titration. Automatic titration with 0.1 M HCL was performed in a recording pH meter (RTS 822, Radiometer, Copenhagen, Denmark). Triplicate runs were made for each sample. From these titration curves, the BC has been calculated for the pH traject 8.0 to 5.2. The BC is expressed in slykes, defined as the number of micromoles of HCl that must be added to 1 ml semen to lower the pH from 7 to 6.

ROLE OF SPERMATOZOA

In order to determine the contribution of the spermatozoa, the semen is centrifuged for 10 minutes at 1200 x g. The spermatozoa are thus precipitated and the supernatant consists of spermatozoa-free semen plasma. The difference of the BC of the original semen and the supernatant semen plasma is the contribution of the spermatozoa to the BC.

ROLE OF HCO_3^-/CO_2 SYSTEM

Of the low molecular weight components the HCO_3^-/CO_2 system might play an important role in the buffering system of semen plasma. This contribution can be established quantitatively by shifting the equilibrium in the direction of CO_2 by dilution and removal of CO_2 from the semen plasma.

First of all, the method of Izutsu[4] is applied: bubbling the fluid with compressed air or oxygen leads to removal of CO_2 and subsequent HCO_3^-. When this procedure is performed at the original pH, the BC of the sample is reduced directly proportional to the length of the bubbling time. Acidification up to pH 5 also leads to CO_2 removal by shifting the equilibrium

towards CO_2. This can be concluded from the difference in amount of acid initially required to titrate the sample to pH 5 and the amount of acid that is needed after backward titration from pH 5 to the original pH with a strong base. By way of this last method, the reduced BC cannot be lowered further by bubbling with oxygen, so the CO_2 removal by acidification to pH 5 must be complete. Therefore, we have only used the acidification method to estimate the contribution of the HCO_3^-/CO_2 system to the BC.

ROLE OF PROTEIN

The charged groups present on protein molecules may contribute to the BC of semen plasma. Trials to estimate this contribution of protein to the BC by removing protein through boiling and subsequent precipitation or acid precipitation have been unsuccessful. However, by means of ultrafiltration through Diaflo ultrafilters (Amicon Danvers, MA), a reliable estimate could be made. The pores of these filters are such that molecules with a molecular weight higher than 10,000 cannot pass the filter, whereas small molecules do pass. A volume of 2.5 ml semen plasma is pipetted in a stirring device in which the ultrafilter membrane has been inserted. The sample has been acidified before with concentrated HCl to remove CO_2. Ultrafiltration is subsequently performed with N_2, pressure 4.5 to 5.0 atmosphere, during 30 minutes at 20°C. Both the BC and the protein concentration of the concentrated semen plasma and the original semen are measured. Assuming that the concentration of other buffering substances, mainly consisting of salts, is equal in concentrated plasma and original semen, it is possible to calculate the contribution of protein to the BC.

The contribution of the various components could be calculated from the values in Table 1. The spermatozoa contribute 1.5% (standard error of the mean [SEM] = 0.1) to the BC of semen and therefore play an insignificant role in the buffering substances. The contribution of the HCO_3^-/CO_2 system has been determined by acidification and subsequent back-titration as described in the materials and methods section. By this procedure the BC is reduced to 75.1% (SEM = 2.5, n = 7) of the original buffering capacity of the complete semen sample. Thus, the HCO_3^-/CO_2 system contributes 24.9% (SEM = 2.5) to the BC of semen. In order to determine the contribution of high-molecular weight components (protein) to the BC of semen samples, the semen plasma samples have first been acidified and back-titrated in order to remove HCO_3^- and CO_2 as described previously. Subsequently the samples have been filtrated through Diaflo filters and both the BC and the protein concentration have been determined before and after filtration. Assuming that the concentration of low molecular weight components in the original sample and the filtrate are equal, the contribution of protein to the BC can be determined by solving two equations. One milligram of protein present in semen has been calculated to have a BC of 0.28 slyke/ml (SEM = 0.02; n = 7). Because the average protein concentration in semen plasma is 45.3 mg/ml, the total contribution of protein is 0.28 x 45.3 = 12.7 slyke, which is 28.5% (SEM = 2.7, n = 7) of the BC of the original semen sample.

DISCUSSION

It is obvious that spermatozoa do not contribute considerably to the BC of human semen. This also has become clear in a previous experimental study[1]: semen samples of men with an azoospermia or oligozoospermia show no significant lower BC than

Table 1. The Quantitative Contribution to the Buffering Capacity of Human Semen of Spermatozoa, HCO_3^-/CO_2, and Protein

sample number	semen	spermatozoa	HCO_3^-/CO_2	protein	
				Per milligram	Total contribution
1	56.3	0.8	15.3	0.26	15.3
2	43.7	0.7	7.2	0.18	8.7
3	48.6	0.6	12.2	0.25	10.3
4	46.7	0.7	12.6	0.30	13.6
5	34.5	0.5	11.0	0.36	10.8
6	45.5	0.7	9.9	0.30	13.7
7	45.9	0.6	9.8	0.30	16.7
8	41.3	0.7			
9	39.8	0.6			
10	43.1	0.7			
mean	44.5	0.66	11.1	0.28	12.7
SEM	1.8	0.03	1.0	0.05	1.1

samples with a normal number of spermatozoa.

It has not been possible to determine the contribution of the bicarbonate system to the buffering capacity by applying the method of Izutsu[4], which was successful for saliva. Bubbling the semen with oxygen does not result in a constant value for the BC. On the contrary, acidification does reveal a constant value. The bicarbonate contribution to the BC of semen is approximately 25%. This is a low percentage in comparison with its contribution to the BC of e.g. saliva: 60% to 90%.[4] The above literature values, however, depend on whether the saliva is stimulated or resting and whether the measurements take place in an open or closed system. Izutsu[4] states that the salivary bicarbonate BC in vivo is that of an open system, which allows CO_2 to escape. The semen depot in the vagina can be considered as a similar situation and has therefore also to be regarded an open system. In our experiments, all bicarbonate has been removed, just as in an open system. However, in most in vitro experiments described in literature,[2,3,4] the bicarbonate buffering contribution has been measured in a closed system. Although the preservation method of the semen samples[1] is trying to minimize the CO_2 escape from the solution preceding the experiments, our data for HCO_3^-/CO_2 BC have to be considered as lower limits.

The contribution of protein to the BC has not been determined before. Experiments of removing proteins by boiling or acid precipitation have been unsuccesful. The ultrafiltration method has enabled us to make a reliable estimation of the protein contribution to the BC: approximately 28%.

The proteins, bicarbonate and spermatozoa together account for approximately half of the total semen BC. Although we have not investigated the role of the other buffering substances in detail, it is likely that other low molecular components, like citrate, phosphate[4] and pyruvate that are present in semen, play an important role.

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CHAPTER V

THE EFFECTS AND FERTILITY RATIONALE OF VAGINAL SODIUM BICARBONATE DOUCHING WITH A 1.5% (WT/VOL) SOLUTION

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(Submitted)

ABSTRACT

Effects of vaginal douching with sodium bicarbonate ($NaHCO_3$), advised in literature in case of low cervical mucus pH in order to improve the post coital test (PCT), were investigated in vitro. The viability of spermatozoa was not affected by moderate hyper- or hypotonicity. Motility however decreased as result of changes in osmolality (OSM), especially under strong hypotonic conditions. These observations support the choice of a 1.5% (wt/vol) $NaHCO_3$ solution for vaginal douching. When the sperm penetration test (SPT)-score was low, in consequence of (1) moderate semen quality, (2) acid cervical mucus or (3) mucus with high viscoelasticity, incubation of the cervical mucus with 1.5% (wt/vol) $NaHCO_3$ resulted in a significant improvement ($P < 0.001$) of the SPT-score (mean, 3.7; standard error of the mean [SEM], 0.2; $n = 26$) compared with control mucus (mean, 2.2; SEM, 0.2; $n = 26$). The viscoelasticity of cervical mucus decreased significantly after incubation with 1.5% (wt/vol) $NaHCO_3$ ($P = 0.01$, $n = 10$). The negative influence of vaginal fluid upon sperm penetrability was apparent from the mean SPT-score, which was significantly lower (3.3; SEM, 0.3; $n = 8$) than

in the control group in which semen was diluted with the same volume 0.9% (wt/vol) NaCl (4.4; SEM, 0.2; n = 8).

INTRODUCTION

Normal interaction between spermatozoa and cervical mucus is generally thought to be critical for the achievement of natural conception in humans. Abnormalities in this process are held to be the explanation for some states of infertility. The postcoital test (PCT) provides essential information concerning this interaction in vivo. Of the various causes which may be responsible for an unfavourable or negative PCT, inappropriate cervical mucus pH has received little attention. Studies of several investigators[1,2] have indicated that the motility of human spermatozoa ceases at a pH below 6. The normal ejaculate readily overcomes the vaginal acidity by its powerful buffering capacity (BC)[3-5]. Since the pH of the endocervical mucus is generally above 6.5 the sperm cells keep up their motility in the mucus once they escape from the hostile vaginal environment. However, when there is a strong vaginal acidification the pH of the ecto- and endocervical mucus may drop as result of the pH lowering effect of the carbohydrate metabolism. A low seminal volume which implicates a low total BC may under these conditions result in an inadequate temporary alkalinization of the vagina, thus rendering the vaginal and endocervical spermatozoa susceptible to a fast return of vaginal acidity. This unfavorable condition can be prevented by vaginal douching with an alkaline solution prior to sexual intercourse. The alkalinization of the vagina after douching is supposed to force a pH rise upon the cervical mucus. Therefore several investigators[3,5,6] advise their infertile patients to douche with a solution of sodium bicarbonate ($NaHCO_3$) when they show a persistently poor or negative PCT, due to acidity of the cervical mucus just prior to ovulation.

The composition of this solution varies from a mixture of 1

table-spoonful of $NaHCO_3$ in 1 quart of tap water[6] to 30 g $NaHCO_3$ per liter[5]. This results in solutions with a $NaHCO_3$ concentration varying from 1.4 to 3% (wt/vol).

Since the spermatozoa are also sensitive to tonicity[7,8], the osmolality of the $NaHCO_3$ douching fluid might be crucial for spermatozoal motility and survival. The vaginal douching with $NaHCO_3$ however has more consequences than only raising the vaginal and cervical mucus pH. It has been shown that dialysis of cervical mucus in vitro against a $NaHCO_3$ solution results in alteration of the composition of cervical mucus[9,10] and in a reduction of viscosity[9], similar to that observed in the preovulatory period. This lower viscosity might, at least in part, be responsible for the efficacy of $NaHCO_3$ douching leading to improvement of the postcoital test in infertile women. Another attributing factor might be the cleaning effect upon the vagina and ectocervix, because vaginal douching removes cellular obstructive elements for the spermatozoa, as exfoliated cells and leucocytes.

We have investigated the effects of different $NaHCO_3$ concentrations upon the viability and motility of spermatozoa in vitro. Thereafter the possible effects of $NaHCO_3$ upon (1) the ecto- and endocervical mucus pH, (2) the viscosity of the cervical mucus, (3) the cleaning of the vagina and (4) the sperm penetrability into cervical mucus in vitro were tested.

MATERIALS AND METHODS

Fresh ejaculates and cervical mucus from fertile and infertile patients attending our clinic have been used in the various experiments.

The osmolality (OSM), measuring the total number of particles per kilogram of solvent, was assessed for several semen samples

and $NaHCO_3$ solutions, using the Osmomat-osmometer (model 030, Salm&Kipp, Breukelen, The Netherlands). The OSM of semen samples was determined at 30 and 60 minutes after ejaculation. The semen samples were centrifugated for 10 minutes at 3500 x g, thus precipitating the spermatozoa. The OSM of the supernatant semen plasma was determined. Furthermore, the OSM of several $NaHCO_3$ solutions, ranging from 0.5% to 4.0% (wt/vol), was measured.

The effect of hyper- and hypotonic $NaHCO_3$ solutions upon the viability, motility and penetrability of spermatozoa was also studied. In these experiments aliquots of 0.5 ml semen were vortexed with 0.5 ml of different $NaHCO_3$ concentrations. An equivalent volume of 0.9% (wt/vol) NaCl was added to control semen samples. In order to judge the viability of the sperm cells a drop of semen -$NaHCO_3$ (NaCl)- solution was mixed with a drop of methylblue/eosin preparation. This mixture was mounted upon a glass slide and subsequently dried by heating. At 400x magnification the viability of 100 spermatozoa was determined (the dead cells staining red) at 0, 60, 120 and 180 minutes after vortexing. The motility of the sperm cells was judged at 37°C and 100x magnification (half blind procedure) 0, 30, 60 and 120 minutes after vortexing. The penetrability of the spermatozoa was assessed by the sperm penetration test (SPT) by means of the capillary tube method described by Kremer[11]. Incubation was carried out for 30 minutes at 37°C. Subsequently the penetrability was judged at 100x magnification by counting the number of spermatozoa after 60, 90, 120 and 180 minutes at 1, 3 and 5 cm from the beginning of the capillary. The penetrability was expressed in the SPT-score of Kremer[11a]. In order to establish the effect of dilution upon the sperm penetration in good cervical mucus aliquots of 0.5 ml semen were vortexed with 0.5 ml 1.5% $NaHCO_3$ (wt/vol) solution. Undiluted semen served as control specimen. The SPT-score was judged after 30, 60 and 90 minutes as described before.

The next experiments were performed in order to establish the impact of $NaHCO_3$ upon the quality of the cervical mucus, expressed in a) the viscoelasticity and b) the SPT-score. For this reason the mucus samples were incubated with $NaHCO_3$ solutions for 30 or 60 minutes at 37°C. The control mucus sample was not incubated. The effect of the concentration of $NaHCO_3$ upon cervical mucus was judged by incubating several mucus samples for 30 minutes with 1.5% and 3% (wt/vol) $NaHCO_3$ solutions. Subsequently a SPT was performed with samples of one single ejaculate. The number of motile spermatozoa was counted after 30 and 90 minutes and expressed in the SPT-score.

The effect of 1.5% (wt/vol) $NaHCO_3$ upon the quality of the cervical mucus was established by means of a SPT-score in three different groups. In the first group semen of moderate quality (score according to Kremer[11b]; 11 to 13 points) was used with mucus of high quality. In the second group good semen (> 13 points) was used with mucus with a pH value < 6. In the third group good semen was used with mucus with increased viscosity. The incubation was performed for 30 minutes. The number of motile spermatozoa was counted after 30 minutes and expressed in the SPT-score described before.

The effect of 1.5% (wt/vol) $NaHCO_3$ upon the quality of cervical mucus samples was also judged by another parameter, viz. the viscoelasticity. The incubation in this experiment was performed for 60 minutes. The viscoelasticity of the cervical mucus was established with a torsion rheometer (Bleeker, Groningen)[12]. In this instrument, the viscoelastic properties were characterized by oscillating a sphere in the sample. An inverse relationship exists between the number of oscillations and the degree of viscosity: the higher the oscillation count, the lower the viscosity.

In order to assess the influence of vaginal fluid upon the sperm penetration into cervical mucus, aliquots of semen samples were mixed with vaginal fluid in a ratio of 5 : 1. These mixtures

were compared with aliquots of the same semen sample that were diluted with 0.9% (wt/vol) NaCl in the same ratio, in a SPT procedure.

RESULTS

All semen samples showed slight hyperosmotic values in comparison with serum 30 minutes after ejaculation (Mean = 336.3 mOsm/l; Standard error of the mean [SEM] = 3.2; n = 20). After 60

Table 1. The Osmolality of different $NaHCO_3$ Concentrations

$NaHCO_3$ (%, wt/vol)	0.5	0.75	1.0	1.3	1.5	2.0	3.0	4.0
Osmolality (mOsm/kg)	109	157	210	280	350	413	600	763

minutes the OSM of the samples was even higher (Mean = 343.1 mOsm/l; SEM = 3.6; n = 20). No difference was found between the OSM in the untreated semen and in the supernatant after centrifugation. The OSM of the different $NaHCO_3$ concentrations in Table 1 shows that the 1.3% (wt/vol) solution is nearly isotonic with serum (280-296 mOsm/l). The spermatozoa seem to be able to resist moderate hypotonic (1 : 1 dilution with destilled water) and hypertonic (1 : 1 dilution with 4% (wt/vol) $NaHCO_3$) conditions as far as the viability is concerned (Table 2).

Even three hours after the semen had been vortexed with any concentration of $NaHCO_3$ or destilled water, the percentage viable sperm cells was not considerably lower than the percentage at time 0 or in the control (0.9% NaCl) sample. On the contrary the motility decreased as a result of changes in osmolality (Table 3A and B). Strong hypo- and hyperosmolality seemed to inhibit both parameters. The decrease of the percentage motile sperm cells was seen immediately after the semen had been vortexed and the diffe-

Table 2. The Percentage Viable Spermatozoa in Mixtures (1 : 1) of Semen Samples (n = 4) and $NaHCO_3$ Solutions of Various Osmolality and Normal Saline after 0, 60, 120 and 180 minutes[a]

	Percentage Viable Spermatozoa							
					% (wt/vol) $NaHCO_3$			% (wt/vol) NaCl
	0.0	0.5	1.0	1.3	2.0	3.0	4.0	0.9
Time (Min)								
0	76.5 ± 5.5	88 ± 3.2	86 ± 2.5	82.7 ± 4.9	84.0 ± 4.0	84.0 ± 5.5	82.0 ± 2.5	79.0 ± 5.3
60	67.5 ± 12.5	88 ± 4.9	86 ± 3.2	72.3 ± 8.5	77.0 ± 5.5	80.2 ± 3.2	82.3 ± 2.5	75.6 ± 3.2
120	69.5 ± 9.5	86 ± 3.2	85 ± 2.5	75.3 ± 6.2	78.5 ± 2.5	79.7 ± 3.2	82.0 ± 3.2	76.5 ± 4.6
180	68.5 ± 12.5	87 ± 3.2	82 ± 4.6	70.3 ± 11.8	80.0 ± 4.0	84.3 ± 5.5	86.4 ± 3.2	79.3 ± 5.3

[a] The data are mean values ± SEM

Table 3A. The Percentage Motile Spermatozoa (Quantitative Motility) in Mixtures (1 : 1) of Semen and $NaHCO_3$ Solutions of Various Osmolality and Normal Saline after 0, 60, 120 and 180 minutes (n = 2)[a]

	Percentage Motile Spermatozoa							
	% (wt/vol) $NaHCO_3$							% (wt/vol) NaCl
	0.0	0.5	1.0	1.3	2.0	3.0	4.0	0.9
Time (Minutes)								
0	27.5 ± 2.5	47.5 ± 2.5	> 60	> 60	> 60	52.5 ± 2.5	52.5 ± 2.5	> 60
60	27.5 ± 2.5	47.5 ± 2.5	> 60	> 60	> 60	35 ± 5	22.5 ± 2.5	> 60
120	12.5 ± 2.5	35 ± 5	> 60	> 60	52.5 ± 2.5	12.5 ± 2.5	5	> 60
180	7.5 ± 2.5	35 ± 5	> 60	> 60	42.5 ± 2.5	12.5 ± 2.5	5	> 60

[a] The data are mean values ± SEM

Table 3B. The Qualitative Motility of Spermatozoa in Mixtures (1 : 1) of Semen and $NaHCO_3$ Solutions of Various Osmolality and Normal Saline after 0, 60, 120 and 180 Minutes (n = 2)[a]

	Qualitative Motility[b]							
	% (wt/vol) $NaHCO_3$							% (wt/vol) NaCl
	0.0	0.5	1.0	1.3	2.0	3.0	4.0	0.9
Time (min)								
0	2.5	4	4.5	5	5	5	5	5
60	3	4	4.5	4.8 ± 0.3	5	4	3	5
120	3	3.5	4.5	4.8 ± 0.3	5	3	2	4.8 ± 0.3
180	2.5	3.5	4.5	4.8 ± 0.3	5	3	2	4.8 ± 0.3

[a] The data are mean values ± SEM

[b] The qualitative motility: 1 = no movements, 2 = bad, 3 = moderate, 4 = rather good, 5 = good and 6 = excellent

rence with the control sample increased with time. The qualitative motility also suffered from strong hypertonic conditions, but the effect of strong hypotonicity was most deleterious. Dilution with 1.0 - 2.0% (wt/vol) $NaHCO_3$ solutions on the contrary, leading to osmolalities ranging from 280 to 380 mOsm/l, seemed to have no disadvantageous influence upon the quantitative or qualitative motility.

The SPT-score showed the same negative effect of hypo- and hyperosmolality. The results are shown in Table 4. Although the SPT-score of the control semen sample in good mucus is initially rather poor, the penetrability of the spermatozoa shows a slight increase with time. This is also the case for the SPT's performed under conditions that differ not very much from isotonicity. There even seems to be a tendency to increasing SPT-scores in the mixtures of semen with 1.0 to 2.0% (wt/vol) $NaHCO_3$, especially after 120 and 180 minutes. The hypo- and hypertonic samples, on the contrary, show instantly a low SPT-score with no enhancement of the penetrability with time.

Incubation of mucus samples with 1.5% (wt/vol) $NaHCO_3$ showed a mean SPT-score of 4.0 (standard error of the mean [SEM]= 0.5, n = 5) after 30 minutes whereas incubation of the same mucus samples with 3.0% (wt/vol) $NaHCO_3$ showed a mean SPT-score of 2.1 (SEM = 0.4, n = 5) at the same time. After 90 minutes these SPT-scores were 4.6 (SEM = 0.7) and 2.3 (SEM = 0.6) respectively. These differences show a tendency to significancy (Signed rank test, P = 0.06). Therefore the next experiments were carried out with 1.5% (wt/vol) $NaHCO_3$.

Figure 1 illustrates the favorable effect of the incubation of cervical mucus with 1.5% (wt/vol) $NaHCO_3$ on the SPT-scores upon certain indications (vide infra). Taking all experiments together the incubated mucus showed a mean SPT-score of 3.7 (SEM = 0.2, n = 26) while the control mucus showed a mean SPT-score of 2.2 (SEM = 0.2, n = 26). This difference is statistically significant (Signed rank test, $P < 0.001$). In the subgroup of

Table 4. The SPT-scores of Mixtures (1 : 1) of Semen and $NaHCO_3$ Solutions of Various Osmolality and Normal Saline in good mucus after 60, 120 and 180 Minutes (n = 16)[a]

	SPT-score[b]						
	% (wt/vol) $NaHCO_3$						% (wt/vol) NaCl
	0.5	1.0	1.3	2.0	3.0	4.0	0.9
Time (min)							
60	2.0 ± 0.3	3.0 ± 1	2.5 ± 0.3	2.8 ± 0.6	2.1 ± 0.1	1.1 ± 0.1	2.4 ± 0.3
120	2.0 ± 0.3	3.3 ± 0.8	2.9 ± 0.3	3.3 ± 0.4	2.0 ± 0.1	1.3 ± 0.3	2.8 ± 0.3
180	2.0 ± 0.3	3.8 ± 0.8	3.3 ± 0.3	3.2 ± 0.4	1.9 ± 0.4	1.1 ± 0.1	3.0 ± 0.3

[a] The data are mean values ± SEM

[b] The SPT-score according to Kremer[11a]: 1 = negative, 2 = bad, 3 = poor, 4 = fair, 5 = good and 6 = excellent

passable semen quality (n = 14) the mean SPT-score improved significantly after the mucus had been incubated with 1.5% (wt/vol) $NaHCO_3$ viz. 3.8 (SEM = 0.3) versus 2.7 (SEM = 0.3) in the not incubated samples (Signed rank test, $P < 0.001$). The mucus samples with low pH values also showed after dilution with 1.5% (wt/vol) $NaHCO_3$ a higher mean SPT score with than without incubation (4.5 SEM = 0.4 vs 1.0, SEM = 0, n = 5). The same was found in the subgroup of mucus samples with high viscoelasticity (2.9 SEM = 0.6 vs 1.9, SEM = 0.4, n = 7). These differences were near significancy (Signed rank test, $P = 0.06$ and $P = 0.05$ respectively). The improvement of the SPT-scores was significantly higher in the subgroup with acid

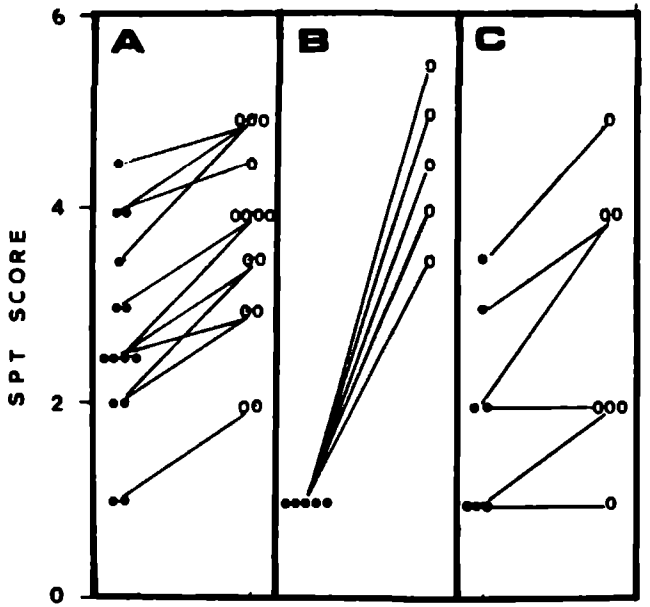

FIGURE 1. The SPT-score of cervical mucus incubated with 1.5% (wt/vol) $NaHCO_3$ (open symbol), versus control not incubated mucus (closed symbol) after 30 minutes. Three indication criteria were present: A Semen of passable quality (n = 14), B mucus with pH < 6 (n = 5) and C mucus with high viscosity (n = 7)

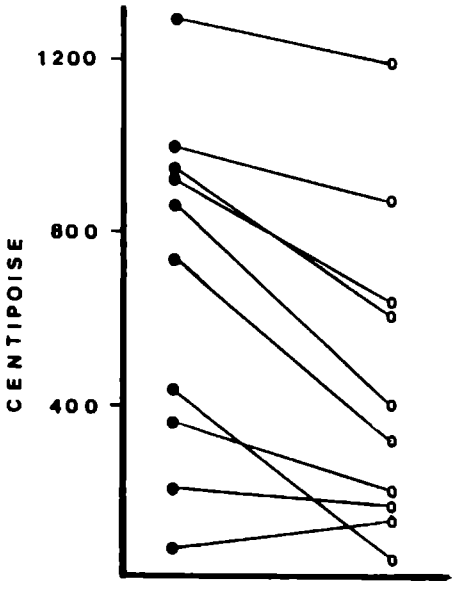

FIGURE 2. The viscosity (in centipoise) of cervical mucus incubated with 1.5% (wt/vol) $NaHCO_3$ (open symbol), versus control not incubated mucus (closed symbol, n = 10)

cervical mucus compared to the other two subgroups (Kruskal Wallistest, $P < 0.01$).

The positive effect of $NaHCO_3$ upon the viscosity of cervical mucus is illustrated in Figure 2. In 9 out of the 10 cases, the viscoelasticity of mucus turned out to be lower after incubation with 1.5% (wt/vol) $NaHCO_3$ (Signed rank test: $P = 0.01$). The negative influence of vaginal fluid upon sperm penetrability is clear from Figure 3. All 8 cases showed a lower SPT score after they had been mixed with vaginal contents. The mean SPT-score in this group was 3.3 (SEM = 0.3, n = 8) versus 4.4 (SEM = 0.2) in the control group. This difference is statistically significant (Signed rank test, $P = 0.01$).

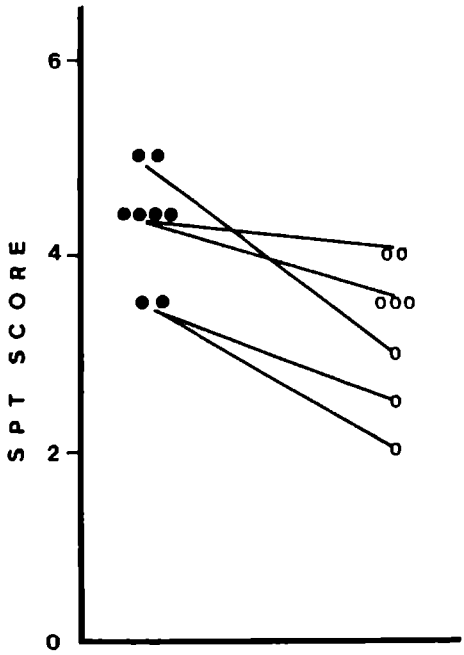

FIGURE 3. The SPT-score of semen diluted (1 : 1) with vaginal fluid (open symbol) versus controle semen diluted with 0.9% (wt/vol) NaCl (closed symbol) in mucus of good quality (n = 8)

DISCUSSION

The results of our measurements of the osmolality[13] of semen samples suggest a rise of the OSM with increasing time after ejaculation. These results are in accordance with those of Makler et al.[8], who have found a mean OSM of 337 mOsm/kg measuring a large number of samples within 1 hour after ejaculation. Semen might even be isotonic during ejaculation[14]. The subsequent rise in OSM could be the result of the activity of proteolytic enzymes, breaking down high molecular weight proteins into smaller fragments. Further investigation of fresh ejaculates with pro-

tease inhibitors is necessary to prove this explanation.

Our data support the importance of the chosen concentration of the $NaHCO_3$ douching fluid. The viability of the sperm does not seem to suffer from hypo- or hyperosmolality, but the quantitative and qualitative motility does. The conclusion of our data and those of Makler et al.[8] is that especially a hypotonic situation seems to be detrimental. Our experiments support the use of a concentration range between 1.0 and 2.0% (wt/vol) $NaHCO_3$ for vaginal douching. Spermatozoa can resist a hypertonic environment better than a hypotonic situation. Semen after ejaculation is slightly hypertonic and sperm velocity is highest when the OSM increases to 400 mOsm/kg[8]. We therefore advise to prescribe the 1.5% (wt/vol) $NaHCO_3$ solution for vaginal douching. The buffering capacity of this solution is 150 slyke, which is of course lower than the buffering capacity of the 3.0% (wt/vol) $NaHCO_3$ solution, but still about 4 times the buffering capacity of normal semen[4]. The potential negative impact of the lower sperm concentration, caused by the diluting effect of douching, might be compensated by its high buffering capacity, especially in cases of low semen volume. This high buffering capacity enables the pH to stay above 6, thus prolonging the time that the spermatozoa can penetrate the mucus[11].

The effect of different $NaHCO_3$ concentrations upon cervical mucus gives extra support for the choice of the 1.5% (wt/vol) solution for vaginal douching. This indicates that infertile couples with persistently abnormal PCT's may profit from this therapeutic measure in case of one or more of the following causal factors: (1) a moderate semen quality; expecially oligozoospermia and low semen volume; (2) acid cervical mucus ($pH < 6$) and (3) mucus with high viscosity.

The data of Jonsson et al.[15] demonstrate that apparent abnormalities of sperm-mucus interaction in vivo, as determined by the post coital test (PCT) may not be confirmed by the in vitro SPT. Many such discrepancies can be attributed to inappropriate timing

of the PCT. This "pseudo cervical hostility"[15] should be recognized and eliminated before precoital alkaline douches are prescribed. As far as acidic mucus is concerned, literature data report that there is no distinct cyclic pattern in cervical mucus pH[5,16-18]. The pH of ectocervical mucus seems in general to be lower than the pH of mucus higher in the cervical canal[5,6,19,20]. This lower ectocervical pH presumably reflects enhanced contamination of ectocervical mucus by acidic vaginal fluids. Alkaline douching removes this acidic source and all the cellular debris, leucocytes and bacteria with it, that may reduce the motility of sperm[21,22]. The fornix posterior can subsequently perform its reservoir function for a few milliliters $NaHCO_3$, thus imitating our incubation experiments in vivo. The last indication for alkaline douching we mentioned was high viscosity of cervical mucus. Since the viscosity of mucus is the greatest barrier to sperm penetration[21] and an inverse relationship between penetrability of sperm and viscoelasticity has been assessed[23], our in vitro experiments create hope for the in vivo situation.

There are however literature data[6,9,10] that support the positive effect of $NaHCO_3$ upon mucus viscosity that we have noted. Gould and Ansari[9] concluded from in vitro testing that there is a predictable effect of electrolytes with monovalent charges on cervical mucus penetrability: the anions increase while cations decrease penetrability. They formulated a plausible hypothesis about the mechanism of action of the charged ions on cervical mucus: exposure of cervical mucus containing negatively charged proteins to salts with multivalent anions causes the protein chains to straighten due to repulsion by the negatively charged anions. In this manner the mucus pore size increases, thereby enhancing sperm penetration. This last effect is consolidated by the presence of only monovalent cations in the salt. $NaHCO_3$ fulfils this condition perfectly. Furthermore, a recent study[24,25] provided another argument to choose for $NaHCO_3$ in vaginal douching. It seems that the bicarbonate anion enhances the motility of the spermatozoa, by stimulating the adenylate

cyclase activity. Thereby, not only an indirect effect of $NaHCO_3$, by alkalinization but also a direct effect upon sperm motility is postulated. So vaginal douching with sodium bicarbonate means more than vaginal alkalinization alone. It enhances sperm penetration in endocervical mucus by neutralizing lactic acid and removal of cellular debris from the vagina and by lowering the viscoelasticity of the cervical mucus. Therefore it can be a meaningful procedure to enhance the chance of conception of those infertile couples with a disturbance of sperm penetration in cervical mucus.

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CHAPTER VI

IMPROVEMENT OF CERVICAL MUCUS VISCOELASTICITY AND SPERM PENETRATION WITH SODIUM BICARBONATE DOUCHING

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ABSTRACT

The aim of the study was to evaluate the influence of vaginal douching with sodium bicarbonate ($NaHCO_3$) upon cervical mucus viscoelasticity and sperm penetration in vitro and in vivo.

Twenty five couples with primary infertility for > 12 months participated in the study. The selection criteria were: (1) semen quality compatible with conception, (2) regular ovulatory cycles and (3) repeated negative post-coital test (PCT). After at least one inventory cycle, three consecutive cycles were studied. In the second and third cycles, vaginal douching was performed with either 1.5% (wt/vol) $NaHCO_3$ or 0.9% (wt/vol) NaCl (randomized procedure). The viscoelasticity of the cervical mucus, sperm penetration tests (SPT) and PCT's were analysed. The viscoelasticity of mucus samples after $NaHCO_3$ douching was significantly lower than the viscosity after NaCl douching ($P < 0.001$, $n = 16$) and in the control cycles ($P = 0.003$).

The SPT scores were significantly higher in the $NaHCO_3$ cycles than in the NaCl cycles ($P = 0.004$, $n = 22$) and in the control cycles ($P < 0.001$). The PCT scores proved to be significantly higher

after $NaHCO_3$ douching than after NaCl douching ($P = 0.002$, $n = 21$).
Comparison of $NaHCO_3$ and control cycles also showed a significant improvement of the PCT score after $NaHCO_3$ douching ($P < 0.001$).

INTRODUCTION

The prevalence of infertility due to disturbed passage of spermatozoa in cervical mucus ranges in literature from 10 to 30% (Steinberg, 1955; Insler et al., 1977; Scott et al., 1977). This diagnosis is based on microscopic examination of post-coital cervical mucus obtained just prior to ovulation in the midcycle. The criteria that the post-coital test (PCT) has to meet to be considered normal are, according to World Health Organization standards: at least seven motile spermatozoa showing forward progression per high power field (x 400) (WHO, 1987). When 'pseudo cervical hostility', as a result of inaccurate timing of the PCT is excluded, several other factors remain that can account for persistently abnormal PCT's. Absence or immobilization of spermatozoa in the cervical canal may be due to unadmitted sexual problems, inability of intravaginal coitus, sperm abnormalities, vaginal infection, ecto- and/or endo- cervicitis, sperm antibodies or mucus problems (Parish et al., 1967; Teague et al., 1971; Jette et al., 1972; Scott et al., 1977). Of course, not all of these conditions will benefit from intravaginal alkaline douchings and have to be solved in another way. However, couples with persistently poor or negative post coital tests as a result of (1) moderate semen quality, especially low semen volume or oligozoospermia, (2) acidic cervical mucus ($pH < 6$) or (3) mucus with high viscosity might benefit from vaginal alkaline douching with sodium bicarbonate ($NaHCO_3$) (Whitelaw, 1979; Ansari et al., 1980; Zavos et al., 1980; Gould et al., 1983).

Although in other studies (Kroeks and Kremer, 1977; Ansari et al., 1980) the composition of the $NaHCO_3$ solution ranged from a mixture of one tablespoonful of $NaHCO_3$ in 1 quart of tap water to

30 g $NaHCO_3$ per liter, in this study a 1.5% (wt/vol) $NaHCO_3$ concentration was chosen because of its (iso)tonicity.

$NaHCO_3$ douching prior to sexual intercourse will neutralize the acidity of the genital tract by its powerful buffering capacity (BC) and will remove most of the vaginal discharge allowing more spermatozoa to become available to the cervical mucus (Teague et al., 1971; Ansari et al., 1980; Wolters-Everhardt et al., 1986). Whether the viscoelasticity of cervical mucus in vivo also can also be reduced and sperm penetration test (SPT) and PCT can be improved by $NaHCO_3$ vaginal douching has been investigated in this study in a prospective double-blind randomized trial.

MATERIALS AND METHODS

Twenty five couples with primary infertility lasting > 12 months participated in this study. The other selection criteria for entering the study were: (1) the quality of the semen sample had to be judged compatible with conception (WHO, 1987), (2) regular ovulatory cycles, if necessary obtained by medication, (3) repeated negative PCT's, (4) unlikeliness of tubal pathology and (5) exclusion of the presence of antisperm antibodies in the serum by means of the mixed antiglobulin reaction test.

One cycle was used for inventory purposes. The growth of the ovarian follicle was monitored by abdominal ultrasound examination from day 8 of the cycle onwards. When the follicle had reached a mean diameter of 14 mm, daily measurements of this dominant follicle were performed until ovulation had taken place (de Crespigny et al., 1981). During these last days prior to ovulation cervical and mucus parameters were assessed according to the criteria proposed by Insler (1977). In this way an impression was obtained of the approximate diameter at which the dominant follicle ovulated and the usual production of cervical mucus by this patient. If necessary, hormonal data were established

after this inventory cycle and medication was administered subsequently to regulate the cycle: bromocryptine in case of (A) hyperprolactinaemia, dexamethasone for (B) hyperandrogenism and clomifene/ nolvadex if neither (A) nor (B) was established. Ectocervicitis was treated by bipolar electrocoagulation or cryosurgery and endocervicitis was treated with antibiotics. If one of these measures was necessary, the inventory cycle was repeated including performance of both SPT and PCT. Subsequently if the couple still met the selection criteria mentioned above, they were included in this study and followed for three consecutive cycles. The first of these three cycles was always the control. In the second cycle vaginal douching was performed with irrigation fluid A and in the third cycle with irrigation fluid B. In order to prepare the irrigation fluids, namely 1.5% (wt/vol) $NaHCO_3$ or 0.9% (wt/vol) NaCl, the patients received a sealed case with two test tubes containing either 4.5 g NaCl or 7.5 g $NaHCO_3$. They were instructed to dissolve the contents of one of the two test tubes in 500 ml boiled and subsequently cooled-down tap water. The sequence of $NaHCO_3$ and NaCl irrigation was randomized and not known by the patients. In the first (control) cycle ultrasound examination was performed and the cervical score was established as described earlier. As soon as the diameter of the dominant follicle approached the dimension just prior to ovulation in the inventory cycle, cervical mucus was collected after cleaning the external os, using a disposable tuberculin syringe. The mucus was stored at 4°C, after sealing the syringe with sterile clay, until the luteal phase. The next day mucus was collected 4-6 h after sexual intercourse, in order to perform the PCT. In the second (investigation A) cycle, when the diameter of the dominant follicle approached the dimension described before, cervical mucus was collected 1-4 h after two vaginal douches with 250 ml irrigation fluid A using a vaginal irrigator (Dagra, Diemen, The Netherlands). The next day, vaginal douching was repeated 1-4 h prior to sexual intercourse. Between 4 and 6 h after coïtus, mucus was collected in order to perform a PCT. The procedure in the third (irrigation B) cycle was the same

as in the second cycle except for the irrigation fluid, which was now fluid B. The investigator who collected the mucus samples did not know the identity of the irrigation fluid. The mucus samples were numbered in sequence of arrival, so that the investigator who judged the samples in the laboratory did not know which couple was concerned and whether irrigation was performed or not.

The viscoelasticity of the mucus collected on the first day of sampling was assessed with a torsion rheometer (Bleeker, Groningen, The Netherlands). In this instrument the viscoelastic properties are characterized by oscillating a sphere in the sample. An inverse relationship exists between the number of oscillations and the degree of viscosity. Furthermore, a SPT was performed using the capillary tube testing system (Kremer, 1965). The mucus was drawn into a capillary tube, one end of which was sealed while the other end was immersed in a fresh sample of partner's semen obtained by masturbation after a period of 2-4 days sexual abstinence. The SPT score was calculated by counting the spermatozoa at three distances (1, 3 and 5 centimeter) along the capillary tubes after 15 and 30 minutes with 100x magnification. The sperm numbers were converted to a one- to seven- point scale (Table I), resulting in six points s_1, s_2, ... s_6. Weighting factors (w_1, w_2, ... w_6) were awarded for the distance travelled by the vanguard sperm (1 cm, x1; 3 cm, x2; 5 cm, x3) and the time in which they covered that distance (15s, x2, 30s, x1). So the highest weighting factor (x6) was given to the sperm that covered the greatest distance (5 cm) in the shortest time (15s). The SPT-score is defined as $w_1 \cdot s_1 + w_2 \cdot s_2 + \ldots\ldots + w_6 \cdot s_6$. The cervical mucus that was collected on the second day of sampling in order to perform a PCT, was judged within 15 min at 37°C in the improved 10 μm counting chamber with a built-in 1 - mm^2 grid (EL - OP, Rehovoth, Israel). In the 100 squares of 0.1 x 0.1 mm^2 each only the motile spermatozoa were counted and among these the proportion of sperm cells showing forward progression (Makler, 1980). The PCT score was calculated by the sum of the locally motile sperm cells and twice the number of the sperm cells showing forward progression.

Table 1.: The Number of Spermatozoa Converted to a one- to seven-Point Scale in the Calculation of the SPT-Score[a]

Point Scale s =	1	2	3	4	5	6	7
Number of Spermatozoa	1-4	5-9	10-14	15-24	25-49	50-99	> 100

[a] see text.

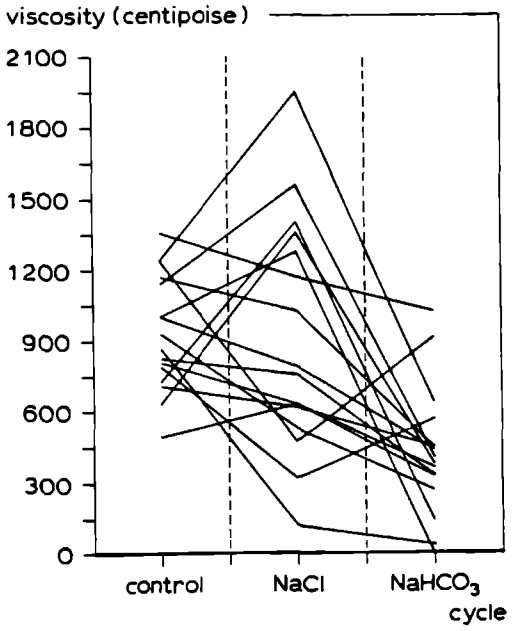

Figure 1. The viscoelasticity of cervical mucus in centipoise in the control, NaCl and $NaHCO_3$ cycles (n = 16)

The control-, NaCl- and $NaHCO_3$ cycles differed significantly (Friedman test) in viscoelasticity measurements ($P < 0.001$, $n = 16$), SPT-scores ($P < 0.001$, $n = 23$) and PCT-scores ($P < 0.001$, $n = 23$). Not all data of these parameters could be obtained in every cycle. In Figures 1-3 and Table II, couples are represented only if the data of all three cycles were available. In order to test mutual differences between the control, NaCl and $NaHCO_3$ data, two cycles at the time were compared for the three parameters mentioned before with the Wilcoxon test for paired cases. The viscosity values of the mucus samples are shown in Figure 1. The viscoelasticity of mucus samples after $NaHCO_3$ irrigation proved to be significantly lower than the viscosity after NaCl douching ($P < 0.001$, $n = 16$) and in the control cycles ($P = 0.003$). The viscoelasticity measurements in the NaCl cycles and in the control cycles differ not significantly ($P = 0.11$).

Figure 2 shows the results of the calculated SPT scores for the three cycles. These data indicate that the SPT scores after $NaHCO_3$ douching are higher than the scores after NaCl douching in 78% of the cases ($P = 0.004$, $n = 22$) and higher than the SPT scores

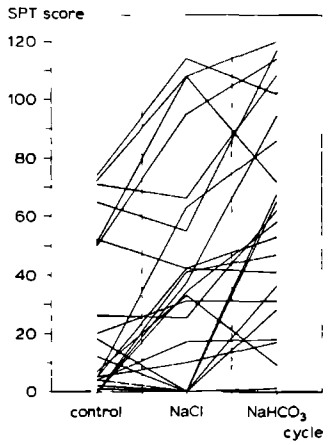

Figure 2. The calculated SPT scores in the control, NaCl and $NaHCO_3$ cyles ($n = 22$)

Table 2.: Viscoelasticity, SPT-score and PCT-score (Mean ± SEM) for the control cycle and the two irrigation cycles. All available samples.

	Cycle		
	control	irrigation NaCl	irrigation $NaHCO_3$
viscoelasticity[1]	874 ± 56	770 ± 112	450 ± 82
SPT-score[2]	22 ± 6	40 ± 8	62 ± 8
PCT-score[2]	1.0 ± 0.4	2.8 ± 1.3	4.8 ± 1.8

[1] in centipoise
[2] see text.

in the control cycles in 96% of the cases ($P < 0.001$). The SPT scores in the NaCl and control cycles also differed significantly ($P = 0.02$, $n = 22$).

The PCT scores (Figure 3) in the $NaHCO_3$ cycles are higher than those in NaCl cycles in 78% of the cases ($P = 0.002$) and higher than those in the control cycles in 88% ($P < 0.001$, $n = 21$). The PCT scores in the NaCl cycles are also significantly higher than those in the control cycles ($P < 0.001$). However, when the PCT percentage score is calculated, i.e. when the number of spermatozoa with local and progressive motility is expressed as a percentage of the total number of sperm cells, there is no statistical significant difference between the NaCl and control cycles ($P = 0.06$), whereas the difference between the $NaHCO_3$ cycles and the other two cycles remains statistically significant ($P < 0.001$).

The overall pregnancy rate, determined 3 months after the study was ended (while the women continued vaginal $NaHCO_3$ irrigation in the preovulatory period as described before) was 20% (5/25). Two pregnancies occurred during the study, both in the $NaHCO_3$ irrigation cycles.

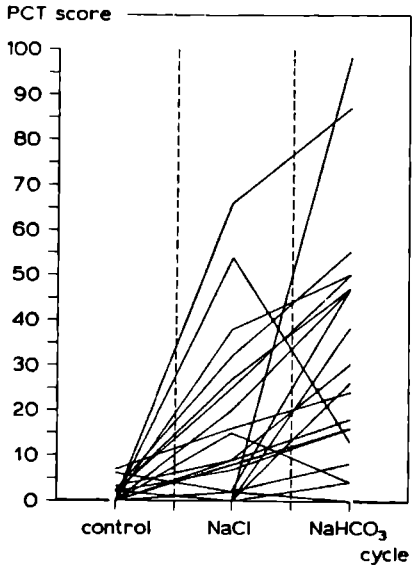

Figure 3. The calculated PCT scores in the control, NaCl and $NaHCO_3$ cycles (n = 21)

DISCUSSION

The principal aim in our investigation has been the appropriate, standardized timing of mucus collection for the performance of the SPT and PCT. We considered that basal body temperature records alone were not suitable for timing ovulation. They are not predictive, but can only be helpful in retrospective analysis, generally indicating ovulation, once the shift has occurred. In addition, the interpretation of these records is submitted to variability in interpretation.

Of the hormonal data, serum estradiol proved to be not useful as an early indicator for prediction of ovulation because of its fluctuations (Garcia et al., 1981). The onset of the LH rise was found to be a more accurate indicator than the LH peak in determining the time of ovulation, but this method requires frequent

serum (or urine) sampling and quick determination (Garcia et al., 1981; Testart and Frydman 1982).

Because of this disadvantage we did not consider the beginning of the LH surge to be a useful parameter in our study. Ultrasound, however, proved in several studies to be a reliable method in detecting and providing positive conformation of ovulation (Wetzels et al., 1982; Vermesh et al., 1987). Ovulation is characterized by a complete disappearance of the transsonic area or by a sharp reduction in size, with appearance of free fluid in the pouch of Douglas (de Crespigny et al., 1981). Ultrasonographic prediction signs described in literature include the appearance of an echodense structure on the inner side of the follicular wall, and a line of reduced reflectivity around the follicle (Picker et al., 1983). The mean preovulatory follicle diameter, however, varies considerably (Renaud et al., 1980). Although no large study is available in the literature concerning the predictive value of ultrasonographically measured follicle size, we have used this parameter, with the other ultrasonographic criteria, for the prediction of ovulation in the individual patient. Two arguments justified this procedure: firstly, all our patients had regular ovulatory cycles and secondly, follicle growth for each patient was extensively followed in a preliminary cycle. We found, like others, that each patient had her own pattern of follicular development (Garcia et al., 1981).

Starting from the ultrasonic ovulation time (UOT: defined as the moment situated halfway between the last observation of an intact follicle and the first observation of the ruptured state) optimal timing would mean mucus collection for SPT performance on day -2 and for SPT on day -1 (Wetzels, 1983). We succeeded in optimal timing in 92% according to ultrasound criteria.

There was no considerable variation in the semen analysis (SA) of each man; 75% showed the same fertility grade in a pair of samples (Kremer, 1968). Therefore, the favourable effect of $NaHCO_3$ upon the SPT and PCT scores could not be the result of better timing of ovulation or a better semen quality in that cycle. Comparison of SPT with different parameters of the SA

revealed that the SPT score is mainly influenced by sperm motility and morfology. This is in accordance with data in the literature (Insler et al., 1979).

We confirmed earlier in-vitro studies which showed that $NaHCO_3$ lowers the viscoelasticity of cervical mucus and improves SPT scores (unpublished data). Douching with 0.9% (wt/vol) NaCl did not result in a reduction of mucus viscoelasticity but nevertheless showed an improvement of the SPT and PCT. These data indicate that the removal of vaginal discharge as a result of vaginal douching probably contributes to the enhancement of the PCT. However, the fact that vaginal douching with $NaHCO_3$ yielded even better PCT scores than irrigation with NaCl justifies the conclusion that the main beneficial effect of $NaHCO_3$ is due to reduction of mucus viscoelasticity, although alkalinization of vagina and cervical mucus by its powerful buffering capacity also has a beneficial effect, especially when the endocervical pH is too low. Despite these favourable results, the only result that really counts for the infertile couple is pregnancy. There is, however, no agreement in the literature about the predictive value of the PCT for pregnancy. Some authors have reported superior pregnancy rates among couples with good PCT results (Moghissi, 1976; Hull et al., 1982; Portuondo et al., 1982), others have been unable to find a similar correlation (Kovacs et al., 1978; Harrison, 1981). This lack of correlation might be due to inappropriate timing, as mentioned before, or to other factors that lie beyond the field of sperm- mucus interaction.

Recently, however, data were published showing the predictive value of the SPT for fertility prognosis (Eggert-Kruse et al., 1989). Our study showed a significant improvement of the SPT score after $NaHCO_3$ irrigation. These data may justifie the conclusion that vaginal $NaHCO_3$ irrigation, upon strict indications, might improve pregnancy chances.

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GENERAL DISCUSSION

The hypothesis that deficient buffering capacity (BC) of human semen is an important determining factor in (male) fertility has to be rejected because of the results of our study. First of all, no correlation has been found between the BC and the fertility grade (1). Secondly, the BC of human semen proves to be much higher than the BC of other human body fluids, as serum, saliva and tear fluid. In the third place even a splitting procedure does not imply essential loss of BC in the first fraction. Therefore the conclusion is justified that the BC of human semen is only deficient in those couples with a persistently negative post coital test because of a low cervical mucus pH despite previous intercourse. In general practice this will only be the case in men with low seminal plasma volume. Experiments with sodium bicarbonate irrigation, originally performed with the intention to buffer the acid cervical environment, showed, by serendipity, a remarkable decrease of the viscoelasticity of cervical mucus. This experience opened a totally new potential indication area for irrigation with $NaHCO_3$, viz. viscous periovulatory cervical mucus.

The periovulatory cervical mucus has several important functional properties:

* receptivity to sperm penetration at or near ovulation and impedence of entry at other times (2,3),
* reservoir function for spermatozoa up to 2-8 days after intercourse (4,5), which is long compared with the duration of sperm motility in the vagina (maximal 0.5 days) and in the uterus and oviducts (2-2.5 days) (4,6),
* supplementing the energy requirements of spermatozoa. Glucose is the major fuel used by human spermatozoa in the female reproductive tract. The affinity for glucose utilization by spermatozoa is nearly 30 times greater than that for fructose, and the

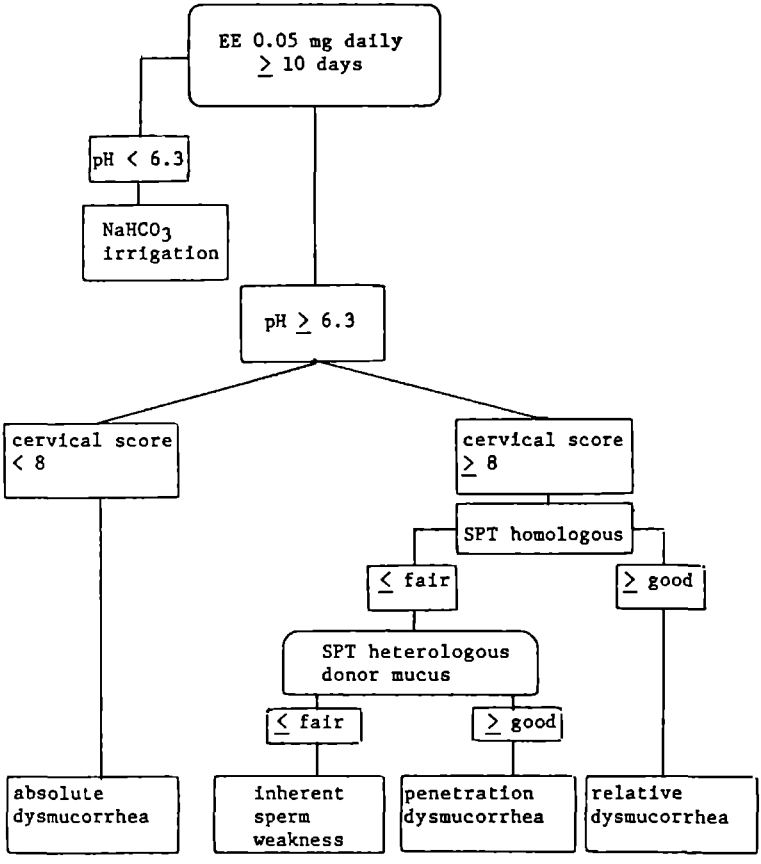
viability of the spermatozoa is directly related to the glucose concentration in the cervical mucus (7,8),

* filtering effect which provides the cervical mucus a pronounced capacity to inhibit selectively the passage of pathologically configurated sperm cells (9),
* protecting the female genital tract from ascending infection through its antibacterial properties (10), and
* possible capacitation of sperm cells.

The sperm migration through the cervix is probably following two distinct phases: the rapid phase takes about 10 minutes in which spermatozoa penetrate the cervical mucus. Some spermatozoa reach the internal os of the cervix within a couple of minutes with a velocity of 0.3-3.1 mm/min (3). The subsequent quick transport, which may be facilitated by increased contractile activity of myometrium and mesosalpinx during intercourse, provides a continuously new supply of spermatozoa, for about 15 to 45 minutes, reaching the upper genital tract (11,12). In the delayed phase massive numbers of spermatozoa colonize the cervical crypts, where a reservoir is formed (12). The protective action of the cervical mucus ensures a constant release of motile sperm into the upper genital tract, following the initial rapid passage, thus increasing the chance of fertilization. The larger the sperm reservoir, the longer an adequate population of spermatozoa will be maintained in the oviduct (12). All these sperm facilitating properties of cervical mucus make perfectly clear, that it is more profitable trying to enhance the quality and/or quantity of cervical mucus, in case of repeated negative PCT's due to relative dysmucorrhea (13), than to pass the cervix and deposit the spermatozoa in the uterine cavity. A flow chart (Fig. 1) modified after Insler et al (13) shows the evaluation of the cervical factor and the position of sodium bicarbonate douching as the only non-hormonal therapy of relative dysmucorrhea.

Decades ago vaginal douching was already used for hygienic and fertility purposes. It was assumed that douching with an acetic acid sclution after intercourse could prevent conception.

Fig. 1 Evaluation of the cervical factor of infertility using pre-treatment ethinylestradiol (EE), estimation of the cervical score (14) and SPT.



When Macleod and Hotchkiss (16) introduced a special medicated douche as a treatment in certain cases of infertility, they restricted its use to the infertility cases of long duration, in which neither partner showed striking abnormalities. This precoital douche consisted of dextrose, calcium gluconate, sodium chloride and potassium chloride. The success of this medication was attributed to temporary removal of some "imcompatibility" that prevented the penetration of spermatozoa into the cervical canal. In the sixties Danezis et al. used this medicated precoital douche to improve the PCT, in which they reported to succeed in 63% (17). Some years later douching with an alkaline solution prior to intercourse was recommended in order to improve the chance upon male offspring (18). Ansari et al. highlighted again douching with an alkaline solution (19), viz. sodium bicarbonate, prior to intercourse, but not for reasons of sex preselection. He advised irrigation in infertile patients with repeated unfavorable PCT's, due to mucus with low endocervical pH or high viscoelasticity, in order to improve sperm survival in the cervical mucus.

The popularity of vaginal douching as a hygienic measure decreased when data from retrospective studies were published, concluding that vaginal douching enhanced the risk of pelvic inflammatory disease and ectopic pregnancy (20,21). These studies however did not supply any information about other potential risk factors as e.g. (multi)parity of the investigated women, promiscuity and frequency of intercourse. Furthermore no information was provided in these studies about the procedure of irrigation and whether it was performed with low or high pressure. The irrigator we prescribe our patients consists of a soft plastic squeeze bottle, on top of which a hard plastic tapering nozzle with side openings has to be screwed after filling (Dagra, Diemen, The Netherlands). The patient has been instructed, verbally and in writing, to insert the tip of the nozzle over a distance of approximately 4 cm in the vagina and to perform the vaginal douching in a sitting position on the lavatory, by softly squeezing the bottle (which means about 5 cm H_2O pressure). Moreover, in the anteverted uterus the plain of the cervical axis

is perpendicular the vaginal axis. This means that the actual pressure upon the cervix is even lower. Furthermore no pressure gradient is built up in the vagina during irrigation because lack of blockade, which offers the irrigation fluid a constant opportunity of returning from the vagina. We considered this low pressure irrigation a safe procedure for our study.

According to Gould and Ansari (22) it is evident that anions increase while cations decrease penetrability. Moreover, it appears that the degree of effect is directly related to the valency of the ion involved. The action of the multivalent ions on cervical mucus is hypothesized as follows: proteins within the cervical mucus are negatively charged and the exposure of the mucus to univalent cations neutralizes such charge. However, when exposed to cations having a valency of two or more, ionic binding occurs among the proteins, with the cations acting as bridges. The pore size within the mucus decreases, thereby inhibiting the penetration of sperm. Addition of multivalent anions to the mucus, particularly with the use of salts in which the cation is univalent, causes the protein chains to straighten due to repulsion by the negatively charged anions along their lengths. In that manner, the pore size within the cervical mucus is increased, thereby enhancing sperm penetration. This theory has been confirmed by scanning electron microscopy. The pore size increasing effect of multivalent anions is consolidated by the presence of only monovalent cations in the salt. Sodium bicarbonate fulfils this condition perfectly. The study of Okamura et al. provided another argument to choose $NaHCO_3$ for vaginal irrigation (23,24). Sodium bicarbonate enhances the motility of the spermatozoa by stimulating the adenylate cyclase activity. Therefore $NaHCO_3$ seems to have not only an indirect motility stimulating effect upon spermatozoa through alkalinization and decline of viscoelasticity of cervical mucus, but also a direct sperm motility stimulating effect.

There are two potential disadvantages associated with vaginal irrigation. First of all the douching procedure can be experien-

ced by the couple as a rather messy affair interfering with spontaneity more than other treatments, that usually implicate taking tablets. The fact however that it concerns infertile couples implies more or less unevitably that they are already fixed upon the periovulatory period, whatever kind of treatment has been prescribed. We do not think that vaginal irrigation will contribute much to the psychological stress of periovulatory intercourse, planned as it is altogether. Another potential disadvantage of frequent vaginal douching with sodium bicarbonate might be a change of the normal bacterial colonization of the vagina, because of continuous pH rise. This persistent alkalinity might increase the risk of especially stubborn mycotic infections which can be a reason, occasionally, to discontinue the treatment. In order to avoid this problem it would be wise to limit the vaginal irrigation to one or at most two occasions in midcycle, which can be achieved with serial ultrasound examinations of follicular growth, or by urine LH determinations.

Prospective studies have generally shown a variable correlation between the PCT result and occurrence of pregnancy (25-31), the maximum pregnancy rate associated with a positive result being 49% (30) and with a negative result 25% (31). The true prognostic value of the PCT is difficult to judge from these studies, however, since the results were not related to time and therefore cannot be compared. The study of Hull et al. (32) has shown that the PCT provides a useful prognosis for fertility. When the test was positive ($\geq$ 1 forward moving spermatozoa per HPF) the chance of conception was fivefold greater than when negative, and only about 10% less than normal. At two years the cumulative pregnancy rate was 84% compared with the peak normal cumulative conception rate of 95% (33). According to the study of Jette and Glass (26) the pregnancy rate was significantly higher when the PCT showed over 20 sperm/HPF than in the group having less sperm per high power field. There is no specific number of sperm in the postcoital test that should be judged as "normal". The finding of even one motile sperm per HPF is reasonable assurance of adequate coital technique and ability of sperm to survive

in the cervical mucus. Last but not least in all pregnancy successes in infertile couples a placebo effect must be considered, as well as an overall therapeutic potential of corrected timing when organizing the PCT, although accurate coital timing has shown to have only little therapeutic value (34).

Recently a prospective study has shown the predictive value of the SPT for fertility prognosis (35). Since our results have shown a significant improvement of the PCT as well as the SPT score, these data may justify the conclusion that vaginal irrigation with sodium bicarbonate upon strict indications might improve pregnancy chances.

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The prevalence of infertility due to disturbed passage of spermatozoa in cervical mucus ranges in literature from 10-30%. The interaction between spermatozoa and cervical mucus can be investigated by means of the postcoital test (PCT), which is already for more than half a century a basic part of any fertility evaluation.

In chapter I the many variables in performance and interpretation of the PCT are described, which explains the difficulty in comparing literature data. The importance of adequately timing is stressed in order to rule out "pseudocervical hostility" as the cause of repeated negative PCT's. The several methods for optimal timing e.g. mucus score, urinary LH determination and ultrasound examination are reviewed. Of the various causes of disturbed passage of spermatozoa in cervical mucus that are mentioned, the importance of an adequate pH, to ensure sperm motility, is emphasized. Literature data upon normal pH values of semen plasma, vagina and cervical mucus are summarized. The buffering capacity of seminal plasma is stressed as an essential mechanism during intercourse to create a vaginal environment compatible with sperm motility. A review is presented of the limited literature data on buffering capacity of animal and human seminal plasma. In order to meet situations of high acidity in the lower female genital tract, vaginal irrigation with sodium bicarbonate has been advocated in literature. This buffering substance is not only reported to enhance the pH but also to create favourable changes in the cervical mucus. The aim of the studies, presented in this thesis, have been delineated.

In chapter II the buffering capacity of human semen samples (n = 270) has been calculated from titration curves. All fertility grades are represented. The average buffering capacity defined as the number of micromoles HCl that must be added to 1 ml semen to lower the pH with 1 unit, has been calculated to be 41.1 slyke in

the physiological range. The maximal BC has been established in the pH range of 5.5 to 6.5. On the alkaline side the buffering capacity declines to about half the maximal value. The BC of human semen proves to be much higher than that of animal semen (bull, buffalo, ram and boar). Compared with other body fluids human semen turns out to have about twice the buffering capacity of serum and thrice that of saliva. Tear fluid has an even lower BC, viz. about 7% of that of human semen. No correlation has been found between the BC of semen and serum of the same man, nor between the BC of semen and the fertility grade. In accordance with literature data a consistent pH rise has been assessed on standing, probably due to loss of some carbon dioxide. We therefore conclude that our BC values represent a lower limit.

In chapter III semen characteristics, pH and BC of first and second fractions of split ejaculates (n = 27) have been established. The spermatozoa show higher concentrations, motility and vitality in the first than in the second fraction, which can be expressed in a 2.2 higher fertility grade of the first fraction. The pH values are equal in both fractions, whereas the mean BC of the first fraction is significantly lower (33.1 slyke) than that of the second fraction (44.6). Since the mean first fraction volume amounts to two third of the mean volume of the second fraction, an ideal splitting procedure may even enhance the difference in buffering capacity. No relationship has been established however between the BC and the fertility grade. We therefore conclude that the lower BC of the first fraction will presumably not be a serious drawback against the use of this fraction in treating some forms of male subfertility. An essential splitting condition however must be that the semen volume is large enough to guarantee that the first fraction has sufficient buffering capacity to overcome the acidic vaginal pH.

Chapter IV describes our investigation concerning the quantitative contribution of several substances of human semen to the overall buffering capacity. The role of the spermatozoa in the

BC proves to be negligible (< 2%). The contribution of the HCO_3^-/CO_2 system, wich has been estimated quantitatively by shifting the equilibrium in the direction of CO_2 by means of the acidification method, amounts to approximately 25%. This is a relatively low share in the BC in comparison with its contribution to the BC of e.g. saliva where it supplies 60 to 90% of the total BC. As in chapter II our data about the HCO_3^-/CO_2 system have to be regarded as lower limits, because of potential CO_2 escape from the solution prior to the experiments. A reliable estimation of the contribution of high molecular weight components (proteins) has been possible by means of ultrafiltration: approximately 25%. It is likely that other low molecular components, as e.g. citrate, phosphate and pyruvate account for the other half of the buffering capacity of human semen.

In chapter V the properties of sodium bicarbonate as douching solution have been investigated in vitro. Since the motility of spermatozoa decreases as result of substantial changes in osmolality, the 1.5% (wt/vol) $NaHCO_3$ solution has been selected for subsequent experiments, as this concentration approximates best the slight hyperosmotic value of semen samples after ejaculation. The buffering capacity of 1.5% (wt/vol) $NaHCO_3$ is still about 4 times the BC of normal semen. The effect of sodium bicarbonate upon the quality of the cervical mucus has been determined through incubation tests by two parameters, viz. the sperm penetration test (SPT)-score and the viscoelasticity. In case the SPT score has been low due to cervical mucus with low pH value or high viscoelasticity, or moderate semen quality, incubation of cervical mucus with 1.5% (wt/vol) $NaHCO_3$ has resulted in a significant improvement of the SPT score. A significant decrease has also been established in viscoelasticity of cervical mucus in incubation experiments. Furthermore the last potential favourable effect of douching with sodium bicarbonate has been evaluated, viz. removal of vaginal discharge. The negative influence of vaginal fluid upon sperm penetrability has been assessed clearly by the significantly lower SPT scores in an experiment in which

semen has been mixed with vaginal contents, in comparison with a control sample which has been diluted with (0.9% wt/vol) sodium chloride.

In chapter VI a clinical study is described which has been performed in order to establish in a prospective double-blind randomized trial the influence of vaginal irrigation with $NaHCO_3$ upon cervical mucus viscoelasticity and sperm penetration in vitro (SPT) and in vivo (PCT). The selection criteria for the infertile couples (n = 25) have been assessed. Ultrasound examination of the dominant follicle as well as cervical mucus scores have been used as parameters for optimal timing the test, the procedure of which has been extensively described. The couples have been followed for three concecutive cycles: in the second and third cycle vaginal irrigation has been performed with either 1.5% (wt/vol) $NaHCO_3$ or 0.9% (wt/vol) NaCl (randomized procedure). The viscoelasticity of mucus samples after $NaHCO_3$ douching has been established as significantly lower than the viscosity after NaCl douching and in the control cycles. Furthermore, both SPT- and PCT scores have shown an evident increase after irrigation, with significantly higher scores for both tests in the sodium bicarbonate cycles than after douching with sodium chloride. When however the PCT percentage score was calculated which means that the number of spermatozoa with local and progressive motility is expressed as percentage of the total number of sperm cells, no significant difference between the NaCl- and control cycles has been established, whereas the difference between the $NaHCO_3$ cycles and the other two remain statistically significant. These data indicate that removal of vaginal discharge presumably contributes to the beneficial effect of vaginal douching. But the fact that $NaHCO_3$ yields significantly better scores than NaCl justifies the conclusion that reduction of mucus viscoelasticity and alkalinization are mainly responsible for the favourable influence of vaginal irrigation in the treatment of dysmucorrhea.

The importance of cervical mucus, enhancing sperm survival and transport to the site of fertilization, has been emphasized in chapter VII. All the sperm facilitating properties of cervical mucus in the periovulatory phase make perfectly clear, that in case of repeatedly negative PCT's due to dysmucorrhea it is more profitable to enhance the cervical mucus quality and/or quantity, than to pass to cervix and deposit the spermatozoa into the uterine cavity. A flow chart for evaluation of the cervical factor and the position of sodium bicarbonate in the therapeutic arsenal is presented. The history of vaginal irrigation, the pro's and contra's have been summarized. A hypothesis based upon literature data about the mode of action of sodium bicarbonate upon cervical mucus and spermatozoa has been elucidated. Finally the potential predictive value of the PCT and SPT for fertility prognosis has been discussed.

Volgens literatuur gegevens is in 10 tot 30% van alle gevallen belemmering van het transport van de zaadcellen in cervixslijm de oorzaak van onvruchtbaarheid. De interaktie tussen zaadcellen en cervixslijm kan onderzocht worden d.m.v. de post coïtum test (PCT), die al meer dan een halve eeuw deel uitmaakt van het routinematige infertiliteitsonderzoek.

In hoofdstuk 1 zijn de verschillen in interpretatie beschreven die er bestaan tussen diverse onderzoekers wat betreft de uitvoering en de interpretatie van de Post Coïtum Test (PCT). Dit verklaart het probleem bij het vergelijken van de verschillende literatuur gegevens. Benadrukt is het belang van de juiste timing teneinde "pseudocervical hostility" uit te sluiten als oorzaak van een bij herhaling negatieve PCT. Er is een overzicht gegeven van de verschillende methodes om de PCT optimaal te timen, o.a. door middel van een scoringssysteem van het cervixslijm, LH bepalingen in de urine en echoscopische follikelmetingen. De verschillende oorzaken van een bij herhaling negatieve PCT zijn genoemd, waarbij het belang van een adequate pH om de motiliteit van de zaadcellen te waarborgen is benadrukt. De literatuurgegevens betreffende de normale pH waarden van semenplasma, vaginavloeistof en cervixslijm zijn samengevat. De buffercapaciteit (BC) van semenplasma is geschetst als een essentieel beschermingsmechanisme van de zaadcellen tegen de zure vaginale pH. Een overzicht is gegeven van de beperkte literatuur over buffercapaciteit van dierlijk en humaan zaad. Vaginale irrigatie met natriumbicarbonaat wordt geadviseerd in de literatuur teneinde een hoge zuurgraad in de vagina c.q. cervixslijm te compenseren. Niet alleen verhoogt $NaHCO_3$ de pH maar tevens zou deze stof een gunstige invloed hebben op de kwaliteit van cervixslijm. De doelstellingen van de studies, die in dit proefschrift zijn beschreven, zijn nader uiteengezet.

In hoofdstuk II is de buffercapaciteit van humane zaadmonsters (n = 270) berekend uit de titratiecurves. Monsters van alle fertiliteitsgraden zijn onderzocht. De buffercapaciteit is gedefinieerd als het aantal micromol HCl dat moet worden toegevoegd aan 1 milliliter semen om de pH met 1 eenheid te verlagen. Deze blijkt gemiddeld 41,1 Slyke in het fysiologische gebied te zijn. De BC is maximaal in het pH gebied van 5,5 tot 6,5. Boven pH 7 daalt de buffercapaciteit snel met ongeveer de helft. Het menselijk ejaculaat heeft in vergelijking met dierlijk (stier, buffel, ram en beer) zaad een veel hogere buffercapaciteit. Datzelfde geldt t.o.v. andere, humane, lichaamsvloeistoffen: de BC van semen is ongeveer tweemaal zo hoog als dat van serum en driemaal zo hoog als de BC van speeksel. Traanvocht heeft een nog lagere buffercapaciteit: deze bedraagt slechts ongeveer 7% van de BC van semen. Er kan geen correlatie worden aangetoond tussen de BC van semen en serum van dezelfde man, en ook niet tussen de BC van semen en de fertiliteitsgraad. In overeenstemming met literatuurgegevens is een consequente pH stijging waargenomen in de monsters enkele uren na ejaculatie, waarschijnlijk ten gevolge van CO_2 verlies. Derhalve hebben wij geconcludeerd dat de berekende BC waarden ondergrenzen vertegenwoordigen.

In hoofdstuk III zijn de verschillende semenkarakteristieken, evenals de pH en BC van eerste en tweede frakties van "split" ejaculaten bepaald (n = 27). De zaadcellen in de eerste fractie blijken in hogere concentratie aanwezig, en tonen een betere levensvatbaarheid en beweeglijkheid, hetgeen tot uiting komt in een hogere fertiliteitsgraad van deze fractie. De pH waarden zijn in beide fracties gelijk, terwijl de BC in de tweede fractie significant hoger is dan in de eerste. Een ideale "split" procedure zou dit verschil in buffercapaciteit nog vergroten, aangezien in onze onderzoeksgroep het gemiddelde volume van de eerste fractie ongeveer tweederde is van het volume van de tweede fractie. Wederom is geen relatie aangetoond tussen de buffercapaciteit en de fertiliteitsgraad. Derhalve is de conclusie getrokken dat de

lagere BC van de eerste fractie vermoedelijk geen ernstig bezwaar vormt om in bepaalde gevallen van mannelijke subfertiliteit alleen deze fractie te benutten. Een essentiële voorwaarde is dan wel dat het zaadvolume groot genoeg moet zijn om te waarborgen dat de eerste fractie, ondanks de lagere BC, toch in staat is om de zure vaginale pH te neutraliseren.

Hoofdstuk IV beschrijft het onderzoek naar de kwantitatieve bijdrage van verschillende substanties in humaan semen aan de totale buffercapaciteit. De rol die de spermatozoa spelen blijkt verwaarloosbaar klein (< 2%). De bijdrage van het HCO_3^-/CO_2 systeem is onderzocht door verschuiving van het evenwicht richting CO_2 door middel van de aanzuringsmethode, en blijkt rond de 25% te liggen. Dit is slechts een geringe bijdrage aan de total BC van semen, in vergelijking met het aandeel van bicarbonaat in de buffercapaciteit van speeksel, nl. 60 tot 90%. Evenals in hoofdstuk II moeten de gegevens betreffende de bijdrage van het HCO_3^-/CO_2 systeem worden beschouwd als ondergrenzen, gezien het mogelijke CO_2 verlies uit de oplossing, voorafgaande aan de experimenten. Door middel van ultrafiltratie is het mogelijk gebleken een betrouwbare schatting te doen over de bijdrage van stoffen met een hoog moleculair gewicht (eiwitten): deze bijdrage blijkt ook ongeveer 25%. Het is waarschijnlijk dat andere componenten in semen met een laag moleculair gewicht als b.v. citraat, fosfaat en pyruvaat verantwoordelijk zijn voor de andere helft van de buffercapaciteit van semen.

In hoofdstuk V worden de verschillende eigenschappen van natriumbicarbonaat in vitro onderzocht en beschreven. Aangezien de motiliteit van spermatozoa afneemt door wezenlijke verandering van de osmolaliteit, is de 1,5% (gew/vol) $NaHCO_3$ oplossing gekozen voor de verschillende experimenten, aangezien deze concentratie het best de geringe hypertone waarde van zaad kort na de ejaculatie benadert. De buffercapaciteit van de 1,5% (gew/vol) $NaHCO_3$ is nog steeds ongeveer vier maal zo groot als de BC van normaal semen. Het effect van natriumbicarbonaat op de kwaliteit

van cervixslijm is onderzocht, door middel van incubatie, met behulp van twee parameters, namelijk de spermapenetratietest (SPT)-score en de viscoelasticiteit. In die gevallen waarin de SPT score slecht uitvalt ten gevolge van cervixslijm met een lage pH of een te grote viscositeit, of door een slechts matige semenkwaliteit, heeft incubatie van het cervixslijm met 1,5% (gew/vol) $NaHCO_3$ geleid tot een significante verbetering van de SPT score. Tevens is gebleken dat incubatie met natriumbicarbonaat aanleiding geeft tot een significante vermindering van de viscositeit van cervixslijm. Tenslotte is de laatste mogelijk gunstige werking van $NaHCO_3$ irrigatie geëvalueerd, namelijk verwijdering van vaginaal desquamaat. De negatieve invloed van vaginale afscheiding op het transport van zaadcellen is duidelijk gebleken uit de significant lagere SPT scores in een experiment waarbij semen is gemengd met vagina-inhoud, in vergelijking met een controle met fysiologisch zout.

In hoofdstuk VI wordt de klinische studie beschreven, die is uitgevoerd om via een prospectieve dubbelblinde gerandomizeerde trial het effect van vaginale irrigatie met $NaHCO_3$ op (1) de viscositeit van cervixslijm, (2) de spermapenetratie in vitro (SPT) en (3) in vivo (PCT) te bepalen. Voor de optimale "timing" is gebruik gemaakt van zowel echoscopische metingen van de dominante follikel als van het scoringssysteem van cervixslijm. De procedures van de irrigatie, de afname van het cervixslijm en de testen zijn uitvoerig beschreven. De echtparen zijn drie opeenvolgende cycli onderzocht: in de tweede en derde cyclus is vaginale irrigatie uitgevoerd met ofwel 1,5% (gew/vol) $NaHCO_3$ of 0,9% (gew/vol) NaCl (gerandomizeerde procedure). De viscositeit van de cervixslijm monsters na vaginale irrigatie met $NaHCO_3$ blijkt significant lager te zijn dan de viscositeit na irrigatie met NaCl of in de controle cycli. Bovendien is een duidelijke verbetering van de SPT en SPM vastgesteld na irrigatie, met significant hogere scores voor beide testen in de irrigatie cycli met natriumbicarbonaat. Bij gebruik van de PCT percentage-score voor evaluatie van de resultaten, waarmee wordt bedoeld het aantal

zaadcellen met beweeglijkheid ter plaatse of progressieve motiliteit uitgedrukt als percentage van het totale aantal zaadcellen, is geen significant verschil aangetoond tussen de NaCl- en controle-cycli, terwijl het verschil tussen de $NaHCO_3$-cycli en de twee overige cycli statistisch significant blijft.

Het belang van cervixslijm, dat de overleving en het transport van zaadcellen naar de plaats van fertilisatie bevordert, is benadrukt in hoofdstuk VII. Al die gunstige eigenschappen van cervixslijm t.o.v. de zaadcellen maken duidelijk dat het in geval van herhaald negatieve PCT's ten gevolge van de dysmucorroe nuttiger is te proberen de kwaliteit en/of kwantiteit van het cervixslijm te verbeteren, dan de cervix te passeren en de zaadcellen intrauterien te deponeren. Een schema is opgezet om de cervicale factor te evalueren en de plaats van natriumbicarbonaat te bepalen in het therapeutisch arsenaal. De geschiedenis van vaginale irrigatie, de voor's en tegen's worden samengevat. Een hypothese gebaseerd op literatuurgegevens aangaande de werkwijze van natriumbicarbonaat op cervixslijm en zaadcellen wordt belicht. Tenslotte wordt de potentieel voorspellende waarde van de PCT en SPT ten aanzien van zwangerschap ter discussie gesteld.

Het in dit proefschrift beschreven onderzoek werd verricht in de vakgroep Biochemie (voorzitter: Prof. dr. J.J.H.H.M. de Pont) van de Katholieke Universiteit te Nijmegen en in de kliniek voor Gynaecologie en Verloskunde (hoofden: Prof. dr. T.K.A.B.Eskes en Prof. dr. R.Rolland) van het Sint Radboud ziekenhuis te Nijmegen.

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STELLINGEN

I

De buffercapaciteit van semen blijkt geen belangrijke factor bij mannelijke subfertiliteit.

II

Vaginale irrigatie met natrium bicarbonaat verlaagt de viscositeit van cervixslijm en verbetert de spermatozoa penetratie test en post coïtum test.

III

Voor een adequate diagnostiek van "focale vulvitis" komt men handen tekort.

IV

De suspensie van de blaashals en die van de blindeindigende vaginatop staan, letterlijk en figuurlijk, haaks op elkaar.

V

Een toevallige ontdekking is geen synoniem voor serendipity.

VI

Het schrijven van deze dissertatie was, ondanks de "multipariteit" en dezelfde deskundige gynaecologische begeleiding, veruit de zwaarste "bevalling".

VII

De uitbreiding van het zwangerschapsverlof tot 16 weken moet slechts worden gezien als een stap in de goede richting.

VIII

Management zou in de opleiding tot basisarts een verplicht vak moeten zijn.

IX

De frustratie van vrouwelijke artsen die steeds "zuster" genoemd worden moet beduidend minder zijn dan de frustratie van broeders die steeds met "dokter" worden aangesproken.

X

"What's in a name".

XI

De mensen zijn minder gevoelig voor geluk dan voor ongeluk "Segnius homines bona quam mala sentire"

Livius

XII

De relatie met leden van de maatschap vergt dezelfde inzet als een huwelijk: eraan werken en investeren is noodzakelijk om scheiding van (operatie-)tafel en (verlos-)bed te voorkomen.

XIII

In alles ontvangt men slechts in verhouding tot wat men geeft.

Balzac

XIV

Glimlachen is de meest elegante manier om je tegenstander de tanden te laten zien.

Werner Finck

XV

Linkshandigheid is geen "contradictio in termines".

XVI

De "eerste" lijn is geen lijn die door de "tweede" lijn getrokken wordt.

XVII

Het voorstel van "rekening rijden" in het kader van de milieuproblematiek voegt weer een geheel nieuwe dimensie toe aan het begrip: "tijd is geld".

XVIII

Als er alleen maar wijzen uit het oosten zouden komen, ...
kon iedereen "mooi help'n drukk'n ".....

vrij naar Herman Finkers