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II

Contribution to the assessment of the minimal number of frozen spermatozoa necessary to obtain fertilization in the cow

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Three concentrations of living sperm (3×10^6 ; 0.750×10^6 ; 0.375×10^6) from frozen semen packaged in straws were studied in a field fertility trial involving 3,222 cows. For each of these concentrations, volumes of 0.20, 0.40, and 0.60 ml were used. The effect of volume of dose and number of sperm it contained on conception rate was estimated by comparing the results obtained from the six series of straws (3 volumes and 3 concentrations) and referring to the results obtained from control doses ($14 \times 10^6/0.40$ ml). Non-return rates obtained with 3 million (3×10^6) spermatozoa did not differ from that obtained with control doses containing 14 million (14×10^6) spermatozoa. Non-return rates up to 63.4 % were obtained with doses containing 750,000 (0.750×10^6) spermatozoa, while doses containing as low as 375,000 (0.375×10^6) spermatozoa fertilized cows in appreciable proportions (58.40 % non-return). These results provide a new contribution to the study of minimal number of spermatozoa necessary for conception. Further, they demonstrate the enormous reproductive potential of a bull used in artificial insemination.

Beitrag zur Beurteilung der für die Befruchtung erforderlichen Mindestzahl an Spermien

R. JONDET

In einem Befruchtungsversuch, der an 3.222 Kühen mit in Pailletten gefrorenem Samen unter Feldbedingungen durchgeführt wurde, wurde die Zahl der nach dem Auftauen lebenden Spermien wie folgt variiert: 3×10^6 ; $0,750 \times 10^6$ und $0,375 \times 10^6$. Jede Spermienzahl wurde mit einer Besamungsdosis von 0,60, 0,40 und 0,20 ml kombiniert. Der Einfluß der Besamungsdosis und der Zahl der jeweils darin enthaltenen Samen-

fäden auf das Befruchtungsergebnis wurde durch Vergleich der mit den sechs Paillettenserien (drei Volumina und drei Spermienzahlen) erhaltenen Ergebnisse ermittelt. Ferner wurden diese Ergebnisse mit denen von Kontroll Dosen (14×10^6) Spermien in 0,40 ml) verglichen. Die non-return-Rate (N.R.), die mit 3 Mio. (3×10^6) Spermien erzielt wurde, unterscheidet sich nicht von derjenigen der Kontroll Dosen mit 14 Mio. (14×10^6) Spermien. N.R.-Raten bis zu 63,4 % wurden mit Dosen zu 750.000 ($0,750 \times 10^6$) Spermien erzielt, während Dosen mit der äußerst geringen Zahl von 375.000 ($0,375 \times 10^6$) Spermien noch einen erheblichen Anteil der Kühe befruchtet haben (58,40 % N.R.). Diese Ergebnisse liefern einen neuen Beitrag zur Beurteilung der für die Befruchtung nötigen Mindestzahl an Spermatozoen. Ferner lassen sie das außerordentliche Fortpflanzungspotential eines Bullen in der künstlichen Besamung erkennen.

CONTRIBUTION TO THE ASSESSMENT OF THE MINIMAL NUMBER OF FROZEN SPERMATOZOA NECESSARY TO OBTAIN FERTILIZATION IN THE COW

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The assessment of sperm number necessary to obtain fertilization in the cow has already been the subject of several studies. Although it has been possible to show a certain relation between the fertility level of a bull, and sperm concentration of the doses of semen injected into cows (2), it seems clear that a reduction to 3 million (3×10^6) living spermatozoa does not seem to affect conception rate (3,4). In a study carried out in 1969 (5), the practical value of a concentration of 3 million (3×10^6) living spermatozoa was again confirmed, while doses of lower concentration (1,500,000 (1.5×10^6) and 500,000 (0.5×10^6), gave lower non return rates (N.R.) which were, however not negligible (63.4 and 51.7 %). For this reason, it was decided to reduce the number of 500,000 spermatozoa further. The present study compares the efficiency of doses of semen containing a number of living spermatozoa as closely as possible to 3 million (3×10^6), 750,000 (0.75×10^6), and 375,000 (0.375×10^6) respectively in variable volumes (0.60, 0.40, 0.20 ml). The control doses contain 14 million spermatozoa in 0.40 ml.

MATERIALS AND METHODS

Two bulls, A and B, of Normandy breed, each one providing 2 ejaculates (or a total of 4) were used. The two ejaculates of bull A, of 6 and 8 ml, give respective concentrations (assessed with the hemocytometer) equal to 1.840 and 1.090×10^9 per ml. Mixed, the total volume was therefore 14 ml and the concentration 1.465×10^9 (mean for 8 successive estimates, 4 per ejaculate). The two ejaculates from bull B, volumes of 7 and 7 ml, had concentrations respectively of 1.415 and 1.025×10^9 per ml. The mixture therefore gave 14 ml of semen of mean concentration 1.220×10^9 spermatozoa per ml.

The first dilution (which corresponded to half of the final volume) was carried out at $+32^\circ\text{C}$, with diluent I (Sodium citrate 2H₂O : 14,7 g.; egg yolk : 250 ml.; distilled water : 750 ml.; penicilline : 1 million I.U.; streptomycine : 2 g.). The temperature was lowered to $+5^\circ\text{C}$ in 30 minutes and, after 2 hours, an equal quantity of diluent II (Sodium citrate 2H₂O : 25,3 g.; egg yolk : 250 ml.; fructose : 20 g.; glycerol : 140 ml.; distilled water : 610 ml.; penicilline : 1 million I.U.; streptomycine : 2 g.), was added in 5 aliquots at 10 minute intervals. The diluted semen was drawn into the straws after 6 hours equilibration. For 0.60 ml, large straws (1.2 ml.) were used the capacity of which was previously reduced to half by altering the position of the internal stopper; the volumes of 0.40 and 0.20, corresponded each one to a quantity of diluted semen which, after filling was actually present in the straws of 0.50 and 0.25 ml respectively.

The freezing was different for each of the three series of doses : it was in fact a function of the volume of the straw used since it is known that the thermal gradient in a cylindrical mass varies with the diameter of the cylinder. The largest doses were therefore frozen according to the technique which has been described at the congress of Trento in 1964, the medium doses according to the method presented at the congress of Paris in 1968, and the small doses according to the process that is described at the present congress of Munich.

- 1 - this study was carried out according to the experimental protocole developed by Professor Ch. THIBAULT to whom I am grateful for valuable assistance, and with the collaboration of J.C. TOURNOUR and Y. RABADEUX for the technical work.
- 2 - SULLIVAN (J.J.) and ELLIOTT (F.I.) - Vith cong. inter. reprod. anim. insemin. artif., PARIS, 1968, vol. II, 1307
- 3 - JONDET (R.) - Vith cong. inter. reprod. anim. insemin. artif., PARIS, 1968, vol. II, 1061
- 4 - STEWART (D.L.) and BERNETT (G.H.) - Vith cong. inter. reprod. anim. insemin. artif., PARIS, 1968, vol. II, 1167
- 5 - JONDET (R.) - C.R. Soc. Biol. 1969, 163, 2678

The constancy of the results obtained when freezing the straws (in a vertical position) in liquid nitrogen vapour appears, among other advantages, as uniformity in cooling. As a result all the doses contained the same number of reanimated spermatozoa after thawing. The preparation of several million doses has shown that mean percentage of survival rate is of the order of 64.0, 65.0, and 66.5, for semen frozen in the 3 types of straws (large, medium, and fine). The dilution rates were therefore calculated as a function of these data and the characteristics of the pure semen collected and, naturally, after freezing, it was ascertained that the content of the doses was effectively comparable to that expected. This verification (mean of 6 estimates, per 2 operators), showed moreover the validity of the protocols chosen : only a few small differences were recorded between the expected theoretical figures and those observed (table I). The semen, distributed amongst 8 inseminators, was used for a total of 3,222 cows, the distribution of which is given in table II. Thawing was carried out immediately before insemination in a water bath at + 35°C (30,15 and 7 seconds for large, medium and fine straws, in that order).

RESULTS AND DISCUSSION

Taking into account the relatively small number of bulls and cows involved, the results obtained are too varied for it to be possible to separate information relative to a possible interaction volume/concentration. Nevertheless, and in spite of the absence of statistical analysis, the results (table II) may show some interesting features :

1 - doses containing 3 million (3×10^6) living sperm after thawing give in general non return rates at 60-90 days which are not beneath those which are obtained with higher concentrations. In absolute value, it is moreover a series of doses of 3×10^6 sperm which gave the best conception rate : 75.74 % in 0.40 ml. These facts further complement results obtained previously (3,4,5).

2 - with few exceptions, fertility decreases with the reduction in the number of sperm per insemination (beneath 3×10^6).

3 - a sperm number of about 750,000 (0.750×10^6) results in a percentage of non return which may reach 63.24 (doses of 0.60 ml).

4 - finally - and this is not the least surprising - doses containing a number of sperm of the order of 375,000 (3.75×10^5) makes it possible to obtain conception rates which are far from negligible (58.40 % for bull A, 53.62 % for bull B, with doses of 0.20 ml).

These results emphasize the enormous reproductive potential of a bull. For the Normandy breed, the mean volume of ejaculate is about 7 ml (mean for 3 216 samples : 7.42 ml), having a concentration which is generally never below 10^9 sperm per ml. In this way it is possible to prepare 1,400 doses per ejaculate, using 5×10^6 spermatozoa per dose to obtain 3×10^6 living sperm after freezing and thawing. For 6 ejaculates per month, or 72 per year, the number of doses per year and per bull may reach more than 100,000. And if, accepting a lower conception rate, doses containing 375,000 living sperm (or 500,000 before freezing) were prepared, it would be possible to increase the production by ten to reach the incredible figure of 1 million doses (1×10^6) per bull per year ! However realizable the operation might be, the least that can be said, is that it would be, without doubt, and genetically speaking, subject to caution.

Bull	Volume of the dose	Survival rate (%)	Total sperm number per dose before freezing	Living sperm per dose after thawing	
				theoretical	actual
A	0.60	65.5	4,687,000	3,000,000	3,070,312
		57.5	1,171,875	750,000	673,835
		52.5	585,937	375,000	307,616
	0.40	72.5	21,538,465	14,000,000	15,615,387
		65.0	4,615,384	3,000,000	2,999,999
		70.0	1,153,846	750,000	807,692
		63.0	576,923	375,000	363,461
	0.20	65.0	4,511,278	3,000,000	2,932,330
		60.0	1,127,819	750,000	676,691
		63.0	563,909	375,000	355,262
B	0.60	65.5	4,687,000	3,000,000	3,070,312
		67.5	1,171,875	750,000	791,015
		63.0	585,937	375,000	369,140
	0.40	67.5	21,538,465	14,000,000	14,538,463
		65.0	4,615,384	3,000,000	2,999,999
		63.0	1,153,846	750,000	726,922
		65.0	576,923	375,000	374,999
	0.20	65.0	4,511,278	3,000,000	2,932,330
		67.5	1,127,819	750,000	761,528
		65.0	563,909	375,000	366,540

Table I - Survival rate and sperm number present (theoretical and actual) in the different series of semen doses.

Number of living sperm after thawing (cf. Table I)	0.20 ml						0.40 ml						0.60 ml					
	Bull A			Bull B			Bull A			Bull B			Bull A			Bull B		
	First	N.R.	Mean	First	N.R.	Mean	First	N.R.	Mean	First	N.R.	Mean	First	N.R.	Mean	First	N.R.	Mean
	Rate	Rate	Rate	Rate	Rate	Rate	Rate	Rate	Rate	Rate	Rate	Rate	Rate	Rate	Rate	Rate	Rate	Rate
	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$	$\frac{\%}{\%}$
14×10^6 control							662	72.50	174	68.96	856	71.77						
3×10^6	182	67.58	75	60.00	257	65.36	184	75.54	73	57.33	204	70.42	237	68.77	67	41.79	304	62.82
$0,750 \times 10^6$	198	54.54	69	56.52	267	55.05	171	53.21	71	57.74	242	54.54	234	63.24	73	56.16	307	61.56
$0,375 \times 10^6$	226	58.40	69	53.62	295	57.28	190	46.84	69	46.37	259	46.71	185	43.24	66	48.48	251	44.62

Table II - Conception results expressed in Non Return rates (60-90 days)

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