Effect of carbon dioxide on the survival of ram spermatozoa frozen by the pellet method

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Summary. Two experiments were conducted to examine the effect of carbon dioxide (CO₂ gas) on the survival of ram spermatozoa frozen by the pellet method. Frozen-thawed semen was flushed with CO₂ gas for various periods and the motility of spermatozoa after thawing was investigated. The longer periods for more than 135 seconds of CO₂ gas treatment significantly ($P < 0.01$) depressed the motility of spermatozoa after thawing. However, flushing with CO₂ gas for less than 90 seconds did not affect the motility. It appears that flushing with CO₂ gas for a limited time, 90 seconds at the time of insemination could be effectively utilized for the rapid method of non-surgical intrauterine insemination with frozen-thawed semen in sheep.

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Introduction

A satisfactory recovery of frozen ram spermatozoa has been obtained. However, fertility of frozen-thawed ram semen has not been good as in the frozen bull spermatozoa, although many workers have reported lambing rates of 55-68%¹⁻⁵⁾. It seems to be that the site of deposition of semen is one of the major factor which affects conception rate following insemination, especially in the ewe. The cervix in the ewe is characteristic due to its great length (4-10 cm) and the complexity of its lumen⁶⁻⁸⁾. Therefore, it has been generally agreed that the site of semen deposition in the ewe is usually at the external orifice of the cervical canal.

Since ANDERSEN et al.²⁾ demonstrated a technique for non-surgical intrauterine insemination in ewes, FUKUI and ROBERTS⁹⁻¹³⁾ have

investigated the effect of non-surgical intrauterine insemination with frozen ram semen on the conception rate. FUKUI¹⁴⁾ and FUKUI and ROBERTS¹⁵⁾ indicated from their experiments that the use of CO₂ gas could be effective for the conventional and rapid method of non-surgical intrauterine insemination in ewes. It is reasonably understood that semen can be forced into the uterus by the pressure of CO₂ gas from as near the external orifice of the cervical canal. This would be a rapid method of deposition due to the removal of the necessity to penetrate the whole length of the cervical canal.

Since MAKISIMOV^{15,16)} reported that CO₂ gas was not harmful to sperm cells, CO₂ gas has been widely used for preservation of ram¹⁷⁾, bull¹⁸⁾ and human spermatozoa (CLIMIE, C. R., 1975, personal communications). In these reports, CO₂ gas has been used as a component of diluents. Therefore, it should be investigated the effect of CO₂ gas on the motility of frozen ram spermatozoa and the period of flushing CO₂ gas for its application

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to non-surgical intrauterine insemination. Because the method of flushing with CO₂ gas is somewhat different from those have been adopted in the previous paper. In our technique, CO₂ gas is flushed at the time of insemination after thawing of frozen spermatozoa and they are exposed for considerably long periods.

Materials and Methods

Two experiments were conducted. Semen was collected from two mature Merino and Border Leicester rams using an artificial vagina. Aliquots of semen were diluted with an extender, *Tris* (hydroxymethyl) aminomethane (300 mM)-glucose (27.75 mM)-egg yolk (15% v/v)-glycerol (5% v/v) and frozen in pellet form according to the method described by FUKUI¹⁹⁾. Frozen pelleted semen were thawed in dry and clean test tubes (2.5 cm in the outer diameter) at 37°C without thawing solution. The thawed semen in the test tube was flushed CO₂ gas for various periods (see Experiments 1 and 2) using a modified 13 gauge needle connected with an inseminating pipette (Fig. 1). The CO₂ gas was flowed with a rate of 2 l/min at 40 cm in water level. The flushed semen was then maintained at 37°C in a water bath. The percentage of motile

Fig. 1. An instrument for supplying CO₂ gas.

spermatozoa assessed under a coverslip on an warm stage (37°C) immediately after thawing, and at the intervals of 2 hours during subsequent incubation up to 6 hours.

Experiment 1: The experiment was designed as follows;

- 1) Breeds of rams: Merino v. Border Leicester
- 2) Replicates: a v. b v. c
- 3) Periods of CO₂ gas treatment (sec): 0 v. 45 v. 90 v. 135 v. 180 v. 225 v. 270
- 4) Incubation (hr): 0 v. 2 v. 4 v. 6

Experiment 2: The experiment was conducted to investigate the effect of CO₂ gas treatment for more rigorous periods on the motility of ram spermatozoa. Factors investigated in the design were as follows;

- 2) Breeds of rams: Merino v. Border Leicester
- 2) Replicates: a v. b v. c
- 3) Periods of CO₂ gas treatment (sec): 0 v. 22.5 v. 45 v. 67.5 v. 90
- 4) Incubation (hr): 0 v. 2 v. 4 v. 6

Table 1. Analysis of variance for the data of Experiment 1

Source of variation	D.F.	M.S.	F
Breeds of rams (A)	1	108.20	3.74
Replicates (B)	2	124.82	4.32*
Periods of CO ₂ gas treatment (C)	6	454.25	15.70***
Incubation (D)	3	3131.73	157.71***
A × B	2	109.53	3.79
A × C	6	94.76	3.28*
A × D	3	149.66	7.54**
B × C	12	70.29	2.43
B × D	6	70.98	3.57*
C × D	18	40.95	2.06
A × B × C	12	28.93	1.00
A × B × D	6	49.07	2.47
A × C × D	18	28.81	1.45
B × C × D	36	12.79	0.64
Error	36	19.86	

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 2. Effect of CO₂ gas treatment on the mean percentage of motile spermatozoa during post-thawing incubation at 37°C (Experiment 1)

Breeds of rams	Motile spermatozoa (%) following various periods of CO ₂ gas treatment (sec)							Means
	0	45	90	135	180	225	270	
Merino	30.3	33.5	33.5	29.8	26.3	25.0	20.6	28.3
Border Leicester	42.0	38.6	32.8	33.4	24.9	33.1	14.3	30.9
Means	36.0	36.0	33.2	31.6	25.6	28.9	17.4	29.6

Table 3. Analysis of variance for the data of Experiment 2

Source of variation	D.F.	M.S.	F
Breeds of rams (A)	1	9.75	1.10
Replicates (B)	2	25.73	2.91
Periods of CO ₂ gas treatment (C)	4	31.69	3.58
Incubation (D)	3	1362.52	229.88***
A × B	3	58.04	6.56*
A × C	4	20.50	2.32
A × D	3	55.32	9.33**
B × C	8	44.30	5.00*
B × D	6	15.71	2.65
C × D	12	15.47	2.61
A × B × C	8	8.85	1.00
A × B × D	6	20.81	3.51
A × C × D	12	15.52	2.62
B × C × D	24	9.58	1.65
Error	24	5.93	

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.**Table 4.** Effect of CO₂ gas treatment on the mean percentage of motile spermatozoa during post-thawing incubation using three replicates (Experiment 2)

Periods of CO ₂ gas treatment (sec)	Motile spermatozoa (%) following three replicates of the sample			Means
	a	b	c	
0	38.0	34.2	41.7	37.9
22.5	38.3	39.9	33.2	37.1
45.0	37.3	36.6	34.2	36.0
67.5	33.6	40.5	38.4	37.5
90.0	27.2	35.3	37.2	33.2
Means	34.8	37.3	36.9	36.3

The data were put into the computer programmes for analysis of variance in both experiments.

Results

Experiment 1: The results of analysis of variance are shown in Table 1. There were highly significant differences in the motility of ram spermatozoa after thawing among the periods of CO₂ treatment ($P < 0.001$) and among the incubation periods after thawing ($P < 0.001$). A significant difference was also found in the replicates ($P < 0.05$). There were significant interactions between the breed of rams and the periods of CO₂ gas treatment ($P < 0.05$), between the breed of rams and the incubation periods ($P < 0.01$), and between the replicates and the incubation periods ($P < 0.05$). Table 2 shows the results of the mean percentage of motility of spermatozoa after various periods of CO₂ gas treatment in 2 breeds of rams. The longer periods for more than 135 seconds of CO₂ gas treatment significantly ($P < 0.01$) depressed the motility of spermatozoa during the incubation at 37°C after thawing.

Experiment 2: The analysis of variance showing in Table 3 indicates that CO₂ gas treatment for less than 90 second did not significantly affect the motility of spermatozoa after thawing, although there were

significant interactions in 3 combinations between the breed of rams and the replicates, between the breed of rams and the incubation periods, and between the replicates and the periods of CO₂ gas treatment. Table 4 shows interrelationship between the periods of CO₂ gas treatment and the replicates in the motility of spermatozoa after thawing. The means of the post-thawing motility slightly declined with three replicates ($P < 0.05$), although it was found no significant differences in the motility of spermatozoa after thawing with either the CO₂ gas treatment or the replicates (Table 3).

Discussion

Although MAKISIMOV^{15,16)} reported that CO₂ gas was not harmful to sperm cells, it is important to determine the period of CO₂ gas treatment on its application for non-surgical intrauterine insemination in sheep. The present experiments indicates that CO₂ gas treatment for less than 90 seconds was not harmful to the motility of ram spermatozoa by the pellet method. In the study of FUKUI and ROBERTS¹³⁾ in situ in the reproductive tract of ewes, CO₂ gas or dye was forced into the uterine lumen for 10 to 30 seconds in almost all cases. At the present time the techniques on non-surgical intrauterine insemination are somewhat time-consuming taking several minutes^{2,12-14)}. The results obtained at the present experiments suggested that flushing with CO₂ gas for a limited time, 90 seconds at the time of insemination could be effectively used for the conventional intrauterine insemination in

sheep without loss of the motility of spermatozoa.

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メンヨウ凍結精液における融解後の精子生存性 に対する炭酸ガスの影響

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FUKUI (1976), FUKUI & ROBERTS (1978) はメンヨウの凍結精液による非外科的子宮内授精について検討し, 炭酸ガスを用いてより迅速な方法を提唱し, その有効性を示唆した。本研究では, 上記術式の実用化に際して問題になると考えられるメンヨウ錠剤凍結精液における融解後の精子生存性に対する炭酸ガスの影響, および炭酸ガスの処理時間について検討したので報告する。

実験方法は, 2 頭のメンヨウ (メリノー種, ボーダー・レセスター種) から人工腔で精液採取し, トリス (300 mM)-グルコース (27.75 mM)-卵黄 (15%)-グリセリン (5%) の希釈液で 5 倍希釈した。冷却および錠剤凍結法は従来の方法によった。37°C で融解後, 試験管内

に 2 l/分で種々の時間 (実験 1:0, 45, 90, 135, 180, 225 そして 270 秒間, 実験 2:0, 22.5, 45, 67.5 そして 90 秒間) 炭酸ガスを吹き込み, その後 2 時間毎に 6 時間までの精子運動率および生存性を観察, 記録した。その結果, 融解後の精子生存性は 135 秒間以上の炭酸ガスの処理で有意に低下した ($P < 0.01$) が, 90 秒以内の処理時間では精子生存性の低下は認められなかった。

結論として, 90 秒以内の炭酸ガス注入は融解後の精子生存性に悪影響はなく, この制限時間内で炭酸ガスを用いて迅速な非外科的子宮内人工授精が実施できるものと思われる。