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BACTERIOLOGICAL STUDY OF RABBIT SPERM AND THE EFFECTS OF ANTIBIOTIC SUPPLEMENTS IN THE CONSERVATION MEDIUM.

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INTRODUCTION

Artificial insemination (AI) is being increasingly used on rabbits in France and in the rest of Europe. Healthwise, reproduction without male-female contact probably eliminates considerable disease transmission.

Yet, in an earlier study on the bacteriology of fresh sperm, we found that AI alone did not prevent the transmission of pathogenic germs. Dangerous germs and/or contaminants are sperm-borne as a result of the AI procedure. We felt, therefore, that it was important to apply a strict sanitary protocol to the whole AI operation, from sperm removal to insemination. We also wanted to test whether contamination could be curbed by adding antibiotics to the sperm. This led to questions on the gradual contamination of sperm mixtures, and the effectiveness and use of antibiotic supplements.

On the other hand, we have not found any relationship between the bacteriological and the biological quality of the sperm. It has not been shown that even highly contaminated sperm collected in an experimental rabbitry could harm the reproductive performance or the health of the herd. But in the near future, broad use is expected of the genetic research that has led to the development of fresh sperm straw, which will be supplied in fresh or frozen form from a limited number of sperm production farms, for use in the rabbit farms. This will create a potential risk of spreading infectious diseases through sperm, a risk heightened by the fact that sperm will be supplied in the form of diluted semen.

Because of this risk, it seemed important to understand the bacteriological change of heterospermic mixtures in time. To do this we applied two protocols: rabbits maintained on wire mesh, and rabbits maintained on straw litter, with heterospermic mixtures diluted in a single conservation medium, with and without an antibiotic supplement.

METHODS AND MATERIALS

Animals and conditions of sperm collection

In our preliminary test, we studied contamination of sperm from various rabbitries and considered that the different in level of contamination was, in the main, due to environmental differences. In the New Zealand meat type rabbit maintained on wire mesh, AI was practiced with maximum hygiene. The rabbit farm looked clearer than the fur and angora rabbit units where animals were kept on straw litter and sanitary precautions were less strict.

We have adopted the same dichotomy as above: two types of housing conditions (wire mesh and straw litter). Sanitary conditions during sperm collection are described for each case.

Housing

Wire mesh. Animals were housed in 138 individual metallic hutches (120 females, 18 males) in three tier batteries. The hutches had circulatory ventilation. Droppings were removed by an automated scrapping system.

Supervision and hygiene were strict, and animals with physical problems were systematically eliminated. The materials and premises were cleaned and disinfected regularly, and every 18 months the battery was thoroughly cleaned and disinfected.

Straw litter. Animals were housed in an area with static air circulation. They were kept in traditional cement hutches on straw litter which was renewed every 15 days when the cages were cleaned. Dropping were removed manually. The herd was rather small, and the selection schedule did not always provide for early culling of sick animals. The area was never completely cleared out and disinfected.

Collection materials and methods

Sanitary conditions, as concerned a) the preparation of the material and, b) the method of sperm collection, were not the same in the two experiments.

Preparation of materials: for the rabbits kept on wire mesh, all the glassware (slides, collection tubes, Pasteur pipets) was sterilised and kept in aluminum foil, away from dust. The artificial vagina (AV) and the neoprene latex caps were carefully rinsed in hot water, put into a detergent, rinsed with distilled water, and then stored in packages in clean polystyrene boxes.

For the rabbits kept on straw litter, the glassware, the artificial vagina, and the neoprene latex caps were washed in hot water, occasionally put in chlorox bleach, dried in an incubator, and stored, without any special protection, in the incubator which was kept in a small laboratory next to the rabbit unit. Disposable slides and Pasteur pipets were used.

Collection method: for the rabbits kept on wire mesh, the AV were filled with hot water and the protection sheaths of the collection tubes were placed in an incubator heated to 44°C, that had been connected the evening before. The slides, the cover slips, the Pasteur pipets, and the collection tubes, in their packing, were heated to 37°C on a hot plate. Immediately after collection, the sperm-filled tubes were placed on a stand, and the biological quality of the sperm was checked.

For rabbits kept on straw litter, the glassware and the sheaths were heated in the incubator at 44°C for 30 min. before collection. The latex cap was placed on the body of the AV which was filled with 45°C tap water just prior to collection. During ejaculation, the AV was in contact with the straw litter.

Regardless of method, the operator did not wear gloves nor clean his hands between sperm collections. He used one AV per male (but not necessarily the same AV for same male each time), and assembled the AV manually.

Sperm processing

For each experiment, a heterospermic mixture using 8 individual sperm samples was prepared. Immediately after collection, the biological quality of the sperm was analyzed, and the mass motility was graded, using the Petitjean scale (1965). Semen graded 7 or above was kept for inclusion in a heterospermic mixture.

Each heterospermic mixture was then divided into two parts and diluted to 1/10th its original strength, in two conservation media, *viz.* TRIS with an antibiotic supplement (1,000,000 IU G sodic Penicilline + 1 g Streptomycine sulphate per liter of TRIS), and TRIS without a supplement.

The TRIS dilution solution contained:

TRIS (Hydroxymethylaminomethane)	30.28 g
D Glucose	12.50 g
Monohydrated citric acid	16.75 g
Double distilled water	to make up 1 liter
Egg yolk	20 ml

Citric acid to set the pH of the final medium at 6.8.

The biological quality of the diluted semen was checked by measuring the individual motility of the spermatozoa and the percentage of live spermatozoa (Andrieu grading scale, 1975).

Bacteriological analysis

The culture media were inoculated with diluted semen at the following hours: 0, 6, 24, and 48. The biological quality of the mixtures was checked again before each inoculation. The bacteriological analysis was used to evaluate the quality and quantity of germs that might have grown in the diluted sperm mixture.

Two methods of inoculation were used simultaneously to detect aerobic and facultative aero-anaerobic germs:

- germ isolation and identification using the öse (platinum loop) method, in which a sample was drawn off by loop from each diluted heterospermic mixture in each conservation medium, and cultured in a Petri dish;

- a count of the total flora in the diluted sperm using a set of dilutions. A specific volume (0.l ml) of each diluted mixture was inoculated by coating the Petri dish.

Two media for culturing the diluted mixtures were used for each of these two techniques, *viz.* Difco tryptose agar + 5% horse serum, and, during the same time period, Columbia agar + 5% horse blood.

The first dish was incubated at 37°C for 24 hr. in aerobic conditions, and the second dish was incubated at the same temperature in an atmosphere enriched with 10% CO_2.

The identification process was mainly based on API System micromethods.

For the Gram negative oxydase positive bacilli, an API 20E plate was used.
For the Gram negative oxydase negative bacilli, API 20E galleries were used, and a nutritive medium (globular extract) was added.
For the Gram positive catalase positive cocci, an API 20 STAPH gallery was used.
For the Gram positive catalase negative cocci, an API STREP gallery was used.

The following media and products were also used:

STAPH LATEX (Difco).
Muller Hinton, Mac Conkey, ornithine mobility, meat liver agar (Bio-Merieux or Pasteur) media.
Tetramethyl-p-phenylenediamine, to search for oxydase.

We did not look for anaerobic bacteria or fungi.

Summary Table

	Wire mesh		Straw litter	
No. of rabbits sampled/ heterospermic mixture	8		8	
No. of heterospermic mixtures	1		1	
Conservation media (Heterospermic mixtures diluted to 1/10th strength and kept at 20°C)	TRIS + antib.	TRIS	TRIS + antib.	TRIS
Hour of inoculation on culture media	0 6 24 48	0 6 24 48	0 6 24 48	0 6 24 48

RESULTS AND DISCUSSION

Biological quality of sperm samples

Rabbits kept on wire mesh (Table no. 1)

The volume of individual sperm samples varied between 0.4 and 0.8 ml. Mass motility was very good since it ranked between 7 and 9 on a 0-to-10 scale. After making a mixture of the sperm collected and diluting it to 1/10th strength, individual motility was measured (on a 0-to-4 scale), and the percentage of live spermatozoa were evidence that:

- at the time of dilution and 6 hours later, motility was very good (3 or 4), most spermatozoa were still alive (95%), and there was no difference between media;

- after 24 hours, motility was still good (3), the live spermatozoa figure had dropped to - a still acceptable - 50-60%, and again, there was no difference between the media;

- after 48 hours, individual motility (3) and the live spermatozoa percentage (50%) were only good in the mixture that had been diluted in the TRIS solution.

For the TRIS + antibiotic test group, motility fell to 1, and only 20% of the spermatozoa were still alive.

Rabbits kept on straw litter (Table no. 2)

Between 0.3 and 0.9 ml of sperm were collected from each rabbit. Mass motility was excellent (between 7 and 9). Like in the case above, readings at 0 hr and 6 hr were very good with individual motility between 3 and 4, and 90-95% live spermatozoa.

But unlike the rabbits kept on wire mesh, readings at 24 hr. were also very good: individual motility was between 3 and 4, with 80% live spermatozoa; media made no difference. At 48 hr. readings dropped for both groups. The TRIS test group had a motility rate of 2, and 40% move spermatozoa. The worst was the TRIS + antibiotics group (motility 1, live spermatozoa 10-20%).

For both groups, the 48 hr. reading showed a time/medium related difference; the residual poor quality of the TRIS + antibiotics group may have been the result of harmful effects from the antibiotic compound.

Bacteriological quality of sperm samples

At the beginning of the experiment, the two conservation media were checked and found to be pathogen-free. Furthermore, bacterial contamination was only appreciable in sperm with total flora counts of over 10^4 germs per ml (Madec 1987). Below this level the sample was deemed not, or only slightly contaminated.

Rabbits kept on wire mesh (Table no. 3)

No pathogens were detected at hr. 0, 6, 24, or 48 in the heterospermic mixture that had been diluted to 1/10th strength in the TRIS + antibiotic conservation medium.

For the other medium, contamination developed along much the same pattern: very slight although detectable at hr. 0 and 6, slightly but clearly more than before at hr. 24, over 10^4 at hr. 48 (and therefore had to be taken into account).

Bacterial identification indicated that the dominant flora were cutaneous germs, namely, staphylococcus epidermidis and corynebacteria, and faecal streptococcus as of hr. 6.

So at worst, this was a facultative pathogenic germ whose origin and vectors may have been introduced through contaminated material and/or manual manipulation.

Rabbits kept on straw litter (Table no. 4).

As for rabbits maintained on wire mesh, germs were not detected at hr. 0, 6, 24, or 48 in the TRIS + antibiotic test group.

For the other medium, the very scant contamination recorded at hr. 0 and 6 rose slightly by hr. 24, and exceeded 10^4 germs/ml at hr. 48. Six different bacteria were isolated in each period. The dominant flora was pseudomonas and aerococcus viridans, but we also found faecal streptococcus, a bacillus, and a colibacillus.

As in wire mesh conditions, there were germs from the environment which were not, or only slightly pathogenic. In this situation the variety was greater because of the straw litter.

CONCLUSIONS

A small size rabbit herd was used in this study, and a very low contamination level appeared in our analysis. This said, we seem to have achieved our initial aim. We especially wanted to confirm what we purported in our preliminary study, *i.e.* that we observed no relation between the biological and the bacteriological quality of the mixture we tested.

As concerns the biological quality of the sperm samples, the antibiotics we tested may have had an adverse effect, but this only became apparent at hr. 48 and, in any case, needs to be confirmed.

Under both test conditions, we found that the biological quality remained very good up to hr. 24, but that for both media degradation was visible by hr. 48.

Concerning the bacteriological count, we would like to emphasize once again that the total flora count was exceedingly low throughout the experiment. This must certainly be connected to the high level of hygiene dictated in the method and maintained throughout sperm collection and the subsequent operations.

This also explains the absence of visible differences in contamination levels in the two experiments, unlike what we observed in the earliest trials, and confirms that there is a risk in using straw litter, but that increased hygiene can minimize the risk.

There is a difference, however, as concerns the variety of germs that were isolated; there were many more in the straw litter experiment.

The main conclusion from this experiment concerns the systematic effectiveness of TRIS + antibiotic as a conservation medium, since no germs were isolated, from beginning (hr. 0) to end (hr. 48), under either test conditions.

Contamination increased with time in the medium where flora was cultured. It was apparent at hr. 24, but was not significant until hr. 48.

As for quality, the germs that were isolated were either not pathogenic or were facultative pathogens that came from the environment (material or manual handling).

The dominant flora varied somewhat, depending on whether the rabbits were kept on wire mesh (staphylococcus and corynebacteria) or on straw litter (pseudomonas and aerococcus viridans). In any case it is interesting to observe the effectiveness of the antibiotic supplement in the TRIS solution on the staphylococcus and the colibacilla since both disappeared from the TRIS + antibiotic group, but were present in the other group.

We are considering expanding the experiment to confirm our results on a larger herd of rabbits.

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TABLE No 1 : BIOLOGICAL QUALITY OF SPERM COLLECTED FROM RABBITS KEPT ON WIRE MESH

Quality of fresh sperm per rabbit			Quality of diluted heterospermic mixture			
No	Volume (ml)	Mass motility	Hour of observation after collection	Conservation medium	Individual motility	Live Spermatozoa (%)
35 063	0.5	8	0	TRIS	4	95
				TRIS + Antib.	3	95
35 062	0.4	9				
35 041	0.6	7	6	TRIS	3	95
35 098	0.4	8		TRIS + Antib.	3	90
35 043	0.8	7				
35 003	0.5	7	24	TRIS	3	50
				TRIS + Antib.	3	60
35 122	0.4	7				
35 091	0.6	8	48	TRIS	3	50
				TRIS + Antib.	1	20

TABLE No 2 : BIOLOGICAL QUALITY OF SPERM COLLECTED FROM RABBITS KEPT ON STRAW LITTER

Quality of fresh sperm per rabbit			Quality of diluted heterospermic mixture			
No	Volume (ml)	Mass motility	Hour of observation after collection	Conservation medium	Individual motility	Live Spermatozoa (%)
21	0.7	9	0	TRIS	4	95
				TRIS + Antib.	3	90
45	0.6	7				
59	0.6	8		TRIS	4	95
61	0.9	9		TRIS + Antib.	3	90
71	0.3	8				
83	0.6	9	24	TRIS	4	80
				TRIS + Antib.	3	80
97	0.3	9				
189	0.4	8	48	TRIS	2	40
				TRIS + Antib.	1	10 - 20

TABLE No 3 : BACTERIOLOGICAL QUALITY OF DILUTED SPERM COLLECTED FROM RABBITS KEPT ON WIRE MESH

Conservation medium	TRIS 1/10		TRIS + Antib. 1/10		Total media	
Hour of observation after collection	Total flora (germs/ml)	No of different bacteria	Total flora (germs/ml)	No of different bacteria	bacteria description	dominant flora
0	220	2	0	0	Staphylococcus epidermidis Corynebactérie	Staphylococcus epidermidis Corynebactérie
6	960	3	0	0	Staphylococcus epidermidis Corynebactérie Streptococcus faecium durans	Staphylococcus epidermidis Corynebactérie
24	8680	3	0	0	Staphylococcus epidermidis Corynebactérie Streptococcus faecium durans	Staphylococcus epidermidis Corynebactérie
48	> 10000	3	0	0	Staphylococcus epidermidis Corynebactérie Streptococcus faecium durans	Staphylococcus epidermidis Corynebactérie

TABLE No 4 : BACTERIOLOGICAL QUALITY OF DILUTED SPERM COLLECTED FROM RABBITS KEPT ON STRAW LITTER

Conservation medium	TRIS 1/10		TRIS + Antib. 1/10		Total media	
Hour of observation after collection	Total flora (germs/ml)	No of different bacteria	Total flora (germs/ml)	No of different bacteria	bacteria description	dominant flora
0	670	6	0	0	Pseudomonas acidovorans Pseudo.maltophila Strep. faecium durans aerococcus viridans Bacillus E. Coli	Pseudomonas Aerococcus viridans
6	580	6	0	0	Pseudomonas acidovorans Pseudo.maltophila Strep. faecium durans aerococcus viridans Bacillus E. Coli	Pseudomonas Aerococcus viridans
24	7760	6	0	0	Pseudomonas acidovorans Pseudo.maltophila Strep. faecium durans aerococcus viridans Bacillus E. Coli	Pseudomonas Aerococcus viridans
48	> 10000	6	0	0	Pseudomonas acidovorans Pseudo.maltophila Strep. faecium durans aerococcus viridans Bacillus E. Coli	Pseudomonas Aerococcus viridans