STUDIES ON PROPERTIES OF THE SEMINAL GEL OF THE BOAR USING NATURAL GEL AND GEL FORMED *IN VITRO*

J. C. BOURSNEILL AND E. J. BUTLER

*A.R.C. Unit of Reproductive Physiology and Biochemistry, Cambridge**
and Houghton Poultry Research Station, Houghton, Huntingdon

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Summary. A method of studying boar seminal gel has been devised which enables investigation to be made on the chemical and physical properties of the gel formed *in vitro* from the bulbo-urethral (Cowper's) gland mucin and either vesicular secretion or seminal plasma. The majority of the haemagglutinin (protein H) in boar vesicular secretion combines with the mucin to form the gel. The content of Zn, Mg and citrate of the natural gel is less (often $0.9\times$) than that of the seminal plasma; this ratio also applies to the Kjeldahl N material imbibed *in vitro* from seminal plasma or vesicular secretion (as distinct from the N contributed by the mucin). The imbibed aqueous solution of seminal plasma or vesicular secretion can be defined as 'interstitial' in that its components are diffusible from the gel matrix into buffer. Part of the interstitial water (possibly as much as 10%) is 'bound' physicochemically to the gel matrix and is unavailable for solvation of the seminal fluid components.

INTRODUCTION

Seminal gel, which is part of the whole ejaculate of boar semen, originates from a combination of the rubbery mucin (CGM), extruded from the bulbo-urethral (Cowper's) gland, with vesicular secretion (McKenzie, Miller & Bauguess, 1938). Involvement with the Proteins A and H in this fluid and in seminal plasma was shown by Boursnell, Hartree & Briggs (1970). If the gel is not separated, it swells by imbibition of fluid, ultimately occupying much of the volume of the total ejaculate. This paper deals with some of the properties of the gel and, more particularly, with the composition of the imbibed fluid in relation to that of vesicular secretion or seminal plasma.

MATERIALS AND METHODS

Collection of materials

Seminal plasma and vesicular secretion samples (twenty-one and three,

* Postal address: Animal Research Station, 307 Huntingdon Road, Cambridge CB3 0JQ.

respectively) were collected from nine boars as described by Bournsnel, Baronos, Briggs & Butler (1972). Four CGM samples were obtained as described by Bournsnel *et al* (1970). Seminal gel was separated about 30 min after the collection of ejaculates.

Experimental treatment of CGM and gel

For the experiments, the CGM (and the gel subsequently formed from it *in vitro*) was held in loops, 3 mm in diameter, made at the end of 8-cm lengths of enamelled copper wire (26 BSW gauge; 0.45 mm in diameter).

To make a loop, the pre-weighed wire was threaded through a cut-off surgical needle which acted as a holder for the wire. The projecting end of the wire was looped round a rod of suitable diameter and twisted upon itself (Text-fig. 1).

With the loop retained in the holder, portions of the CGM (about 20 mg) could be scooped up and formed into an adherent mass by dextrous turning of the holder and wire. The CGM and wire were removed from the holder and weighed on a support which avoided contact of the CGM with the balance pan.

TEXT-FIG. 1. Diagram of enamelled copper wire loop held in surgical needle with cut-off tip (scale in cm).

Samples (about 100 mg) of 'natural gel', i.e. gel formed *in vivo* although the actual formation was not studied, were cut with scissors and impaled on similar pieces of unlooped wire.

With this method, the samples of CGM or gel could be suspended freely in seminal fluid or buffer, avoiding contact with the sides of the vessel. It was often found convenient to use Plasticine (Harbutt's Plasticine Ltd, Bath, England) to fix the free upper end of the wire without movement.

Immersion of the CGM in seminal plasma or vesicular secretion caused the formation of the gel which adhered to the loop, making it possible to carry out analyses on known weights of the CGM or gel treated in various ways, e.g. by washing in buffers. The weight of the gel still attached to the wire was determined after quickly blotting on filter paper. The free fluid was easily removed and further weight loss was negligible in the interval required for weighing. Kjeldahl N determinations as described by Bournsnel *et al.* (1970) could be carried out with about 1 cm of the wire still attached to the CGM or gel. The N content of the interstitial fluids (as defined in this work) could be determined by subtracting the N value calculated for 20 mg CGM from the N value of the gel formed *in vitro*, also corrected to 20 mg initial weight of CGM. A similar but not identical value could be derived by multiplying the weight increase by the N value of the seminal fluid used.

Buffers

The buffers used for washing were, except where otherwise indicated, at pH 7, $I = 0.3$ (Miller & Golder, 1950).

Determinations of substances

Haemagglutinin titration of protein H followed the method of Boursnell (1967), using sheep red cells. Zinc, calcium and magnesium were determined by atomic absorption after dry ashing or by the Kjeldahl method (for Zn) as described by Boursnell *et al.* (1972). Ergothioneine was determined by Mann's (1964) modification of Hunter's (1949) method, and citrate by the method of Speck, Moulder & Evans (1946) as modified by Mann, Davies & Humphrey (1949).

RESULTS

Effect of dialysis of vesicular secretion on gel formation

Gel formation from CGM was studied with (1) native vesicular secretion, (2) 2 ml vesicular secretion dialysed against 200 ml buffer and (3) a small volume (4 ml) of buffer which had been used to dialyse 2 ml vesicular secretion. Normal gels formed in the first two cases but in the third, a sticky, rather runny gel was formed.

Weight and nitrogen gain during formation of gel

Treatment of CGM for 24 hr with vesicular secretion or seminal plasma caused rather similar mean weight gains (e.g. vesicular secretion, $4.8 \times \pm 0.17$ S.E.M.; seminal plasma, $5.6 \times \pm 0.12$ S.E.M.). In another experiment with different samples, the mean weight gains after 22 hr were $4.92 \times$ (vesicular secretion) and $5.40 \times$ (seminal plasma); a further 8% was gained by both after 22 hr subsequently in buffer. These experiments showed an interstitial N concentration about 80 to 90% of that of the treating seminal fluid. Rapid loss of N occurred when gels formed *in vitro* were washed in buffer; typically, 19% was lost in 10 min and 33% in 2 hr. Further N loss was small; at 24 hr, it was not significantly different from that at 3 hr (comparable N losses – 38% in 22 hr – were encountered when natural gel samples were similarly washed).

The stable N values in the *in-vitro* experiments were, after dialysis, greater than the CGM N alone. Loss of N was not due to a dialysable N compound (e.g. a polypeptide) released by the formation of the gel; no N was lost on dialysis of gel formed from already dialysed seminal plasma and CGM when these were subsequently combined inside a cellophane sac.

The weight gain of CGM samples in seminal plasma was followed at short intervals (10 min) and related to the increase in the total and interstitial N of the gel. The N per unit weight of the gel (total and interstitial) remained substantially constant in spite of a curvilinear weight increase to $2.9 \times$ that of the initial CGM. After 1 hr, neither the weight increase nor the N gain had apparently ceased. The ratio of observed N to 'seminal plasma N plus CGM N' was constant (0.96 ± 0.01 S.E.M.) throughout. Samples of CGM were treated with seminal plasma for even shorter intervals (10 sec, 20 sec, and 1, 5 and 10

min) and analysed for N with or without a period (24 hr) in buffer. Untreated CGM dispersed in buffer but CGM samples treated with seminal plasma, even for 10 sec, were frail but manageable. With increasing time, firmer gels resulted. In no case was interstitial N detected although the weight gain was about $2 \times$ in all, rising to 6% in the buffer. At periods up to 1 min, the N values of the gel formed were less (about 13%) than those of the untreated CGM; the frail gels were seen to lose uncombined CGM during transfer. Buffer washing removed N

TEXT-FIG. 2. Interstitial nitrogen (—) and gain in weight of gel (---) per 20 mg initial Cowper's gland mucin (CGM) after its treatment with doubling dilutions of boar seminal plasma (○ and ●) or boar vesicular secretion (□ and ■). (N = neat seminal fluid). Alternative calculations (per 20 mg initial CGM) of 'interstitial' nitrogen from corresponding seminal plasma or vesicular secretion nitrogen values multiplied by (gain in weight - 1).

from the samples which had short treatment times (49% at 10 sec, 74% at 30 sec and 72% at 1 min) but at 5 and 10 min, the loss was negligible. Gel formed *in vitro*, with properties resembling the naturally formed gel, took several minutes to mature at room temperature (19° C).

Effect of dilution of seminal plasma and vesicular secretion

Serial doubling dilutions in buffer of 2 ml seminal plasma (3.46 mg N/ml) and vesicular secretion (12.8 mg N/ml, i.e. $3.7 \times$ the seminal plasma concentration)

were used to form gels from CGM. After 23 hr, although a looser gel was formed at the higher dilutions, it was possible to examine (Text-fig. 2) the gain in weight at dilutions of 1/128 with seminal plasma and 1/1024 with vesicular secretion. This gain was almost constant (about $5 \times$) up to 1/8 (seminal plasma) and 1/32 (vesicular secretion), i.e. at similar seminal fluid N values (about 0.4 mg N/ml). Beyond this dilution, a sharp, approximately linear, increase occurred.

The difference between interstitial N and the N content of the corresponding diluted seminal fluids was relatively constant (seminal plasma, neat to 1/32, mean = 0.030 ± 0.005 S.E.M.; vesicular secretion, 1/4 to 1/128, mean = 0.041 ± 0.01 S.E.M.).

Effect of gel formation on haemagglutinin titre of seminal plasma

The haemagglutinating titre of 0.3-ml quantities of seminal plasma was reduced from 4096 to 512 by a 4-hr immersion of a CGM sample in each. This represents an eightfold reduction in the haemagglutinin, but the gel only absorbed about 20% (mean 59 mg) of fluid from the seminal plasma. Interstitial N was 90% of the seminal plasma value, and 45% of this was removed by washing in 0.3 ml buffer. The resulting titre of this buffer (128, or one quarter of the treated seminal plasma) is consonant with the 1/6 dilution (59 mg diffused into 300 mg buffer) of the free interstitial seminal plasma.

Effect of temperature on gel formation

The effect of temperature on gel formation was studied when CGM samples were immersed for 1 hr in 2 ml vesicular secretion at 4°, 19° and 37° C. The weight gain increased with temperature: at 4° C, $1.9 \times$; at 19° C, $2.3 \times$; at 37° C, $3.1 \times$. After washing for 2 hr with 200 ml buffer at 19° C, the gain further increased: at 4° C, $3.0 \times$; at 19° C, $3.3 \times$; at 37° C, $3.6 \times$. Interstitial N (mg/ml) also rose with temperature: at 4° C, 0.204; at 19° C, 0.298; at 37° C, 0.524. This increase was evident even when allowance was made for the extra weight gained at 19° C and 37° C relative to that at 4° C: at 4° C, 0.204 (as above); at 19° C, 0.251; at 37° C, 0.328. Washing showed that the increased N (again adjusted to constant weight gain) was 'fixed', the amounts lost being substantially constant at all temperatures: at 4° C, 0.189; at 19° C, 0.210; at 37° C, 0.184. At all temperatures, the concentration of interstitial N (mean 12.0 ± 0.6 S.E.M.) represents about 90% of the vesicular secretion value.

Influence of ionic strength

The influence of ionic strength (I) of the buffer was studied when samples of gel formed *in vitro* were washed in buffers at seven values of I from 0.2 to 0.5, inclusive. Both the mean weight gain ($5.58 \times \pm 0.11$ S.E.M.) and the interstitial N remaining ($31.9\% \pm 4.1$ S.E.M. calculated on the seminal plasma value) were constant at all values of I .

Distribution of seminal zinc in gel, seminal plasma and spermatozoa

The distribution of zinc between seminal plasma, spermatozoa and gel was studied in three ejaculates. After determination of the weights or volumes of the

Table 1. Zinc in seminal plasma, gel and spermatozoa of total boar ejaculates

Animal and date of collection	Wt or vol. of sample*			Total Zn content of sample (μ g)			Concentration of Zn in sample		
	Seminal plasma (ml)	Gel (g)	Spermatozoa (g)	Seminal plasma	Gel	Spermatozoa	Seminal plasma (μ g/ml)	Gel (μ g/g)	Spermatozoa (μ g/g)
F 8 June 1970	285 (77.5)	75.76 (20.6)	6.80 (1.9)	2205 (65.5)	409 (12.1)	755 (22.4)	7.7	5.4	111
F 11 June 1970	270 (74.76)	86.89 (24.0)	5.19 (1.4)	1223	Lost	369	4.6	Lost	71
E 11 June 1970	190 (78.8)	47.56 (19.7)	3.49 (1.5)	4843 (78.6)	995 (16.2)	321 (5.2)	26	21	92

Figures in parentheses represent percentages of total weight of sample or zinc in whole semen.

* Wet weights.

entire phases, they were separately freeze-dried. The products were weighed, triturated and equilibrated to air humidity. Weighed samples were analysed for Zn (Table 1). The zinc concentrations in the two available gels were 70 and 81% of the corresponding seminal plasma values; other direct comparisons of Zn between natural gel and seminal plasma samples gave values of 79% and 57%. Magnesium (86% and 88%) behaved similarly, but the Ca values considerably exceeded (about 2.5 to 4 ×) those of the homologous seminal plasma samples: Ca values found in CGM samples were very high (mean, 360 µg/g wet weight).

Dialysis of 4-g samples of natural gel at 4° C in buffer at pH 5 slowly removed zinc (79% at 48 hr; 92% at 96 hr). The resulting gels were as opalescent as the *in-vitro* preparations. In other experiments at pH 6, 7 and 8, the amount of zinc removed appeared to lessen with increasing pH.

Citrate, found in 15% (w/v) trichloroacetic acid in which weighed samples of natural gel from two whole ejaculates had been immersed for 12 hr at 19° C, corresponded to 70% and 51% of that in the homologous seminal plasma. In other experiments, both citrate and ergothioneine were found to be readily diffusible from natural gel into buffer at pH 7.

DISCUSSION

The formation of the gel from CGM, incorporating much of the Protein H in vesicular secretion, is a process which takes some time to mature. Bournsnel *et al.* (1970) showed that, with 5% (w/v) solutions of CGM, initial gel setting was very fast, but this is probably an initial stage in a process which is only completed after some time. The formed gel imbibes seminal plasma or vesicular secretion components, some of which reflect the composition of the seminal fluid and are diffusible into a buffer. Slow maturation of the gel may be due to slow diffusion of these components through the partly formed matrix of the primary gel. With increasing temperature, more N-containing material becomes fixed in the gel structure and is non-diffusible. With more dilute seminal plasma or vesicular secretion (protein N about 0.4 mg/ml), a probable structural change results in a weakened gel. The partial formation of the gel with the buffer used for dialysing the vesicular secretion suggests a similarity to the ultrafiltrate, containing traces of Protein A but not the haemagglutinin, described by Lavon, Bournsnel & Briggs (1972) and Bournsnel, Briggs, Lavon & Butler (1973).

The interstitial composition of the gel formed *in vitro* appears to be akin to the naturally formed material, although the latter exhibits spherical discontinuities within the round aggregates. These appear to be slowly eliminated by further swelling in buffer, resulting in an opalescent homogenous gel resembling the material formed *in vitro*.

The non-dialysable N values in the experiments carried out *in vitro* were greater than the CGM alone and this suggests that, in addition to loosely held interstitial nitrogenous components, there was a 'fixed' part incorporated more closely into the gel molecular structure. It was shown that the loss of Protein H was due to its selective incorporation into the matrix of the gel as a fixed part of the interstitial N as defined above. Once fully formed, the continued presence

of the gel in semen did not alter the titre of seminal plasma samples separated at intervals (Bournsnel, 1967).

Part of the purpose of this work was to discover if the gel specifically incorporated Protein H and Zn. The ratio of the concentrations of these two substances in seminal plasma and vesicular secretion was lower than with other materials (about 0.2) examined by Mann & Glover (1954): the haemagglutinin titre of seminal plasma is often five tubes lower than that of vesicular secretion, representing a 32-fold dilution (i.e. 2^{-5}): the zinc ratio, taken from the mean figures given by Bournsnel *et al.* (1972), is 0.165. It was shown that the loss of Protein H is due to its incorporation into the gel structure. The Zn was not thus incorporated, but the high Zn values in spermatozoa suggest that part at least may be absorbed from the vesicular secretion and this aspect is being investigated.

The values for Ca in CGM found in this work are greatly in excess of the low Zn values reported by Bournsnel *et al.* (1972) and could account for almost all the extra Ca concentration encountered in the gel compared with the homologous seminal plasma. This Ca may help to neutralize the sialyl residues in CGM (Hartree, 1962).

In many cases in this work, nitrogen and other values for interstitial fluid were about 80 to 90% of that of the corresponding seminal fluids. It is suggested that part of the reason for these low values may be due to the presence of 'bound' or 'combined' water in the gel, unavailable for dissolving solutes (Moran, 1932).

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