Identification of perivitelline N-linked glycans as mediators of sperm–egg interaction in chickens

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This study demonstrates that carbohydrates play an essential role in sperm–egg interactions in birds. Sperm–egg interaction was measured in vitro as the ability of spermatozoa to hydrolyse a small hole in the inner perivitelline layer, the equivalent of the mammalian zona pellucida. Preincubation with Triticum vulgaris lectin (WGA) and succinyl-WGA (S-WGA) at 10 μg ml⁻¹ resulted in complete inhibition of sperm–egg interaction, whereas at the same concentration a range of other lectins (Canavalia ensiformis (Con A), Arachis hypogea (PNA), Ulex europaeus II (UEA II), Solanum tuberosum (STA), Tetragonolobus purpureus (LTA) and Pisum sativum (PSA)) were unable to inhibit sperm–egg interaction significantly, although fluorescein-labelled derivatives of these lectins were found to stain the inner perivitelline layer. Significant inhibition of sperm–egg interaction was achieved by the addition of N-acetyl-d-glucosamine and fucoidin to the assay mixture; however, d-glucose, d-galactose, d-fucose and l-fucose had no significant effect on sperm–egg interaction. Pretreatment of the inner perivitelline layer with N-glycanase significantly reduced sperm–egg interaction, whereas treatment with O-glycanase had no effect. These results demonstrate that N-linked glycans play an essential role in sperm–egg interaction in chickens.

Introduction

Fertilization involves binding and fusion of gametes. In mammals (for reviews see Yanagimachi, 1994; Benoff, 1997; Shalgi and Raz, 1997; Wasserman, 1999) and some non-mammals, including ascidians (Rosati and De Santis, 1980) and sea urchins (Kinsey and Lennarz, 1981), there is considerable experimental evidence to support the view that the initial stages of gamete interaction are carbohydrate-mediated events. Competitive inhibition studies that used various lectins, monosaccharides, polysaccharides or glycoproteins have demonstrated a role for specific carbohydrate moieties in mediating sperm–zona interactions in several mammalian species, including hamsters, humans, guinea-pigs (Huang et al., 1982), rats (Shalgi et al., 1986) and mice (Shur and Hall, 1982; Lopez et al., 1985; Bleil and Wasserman, 1988; Cornwall et al., 1991).

In birds, as in mammals, fertilization occurs internally. However, the avian egg at ovulation is much larger than the mammalian egg and it is surrounded by a glycoprotein layer known as the inner perivitelline layer (Bellairs et al., 1963; Kido et al., 1975; Kido and Doi, 1988), which is similar to the zona pellucida and acts as an initial binding site for spermatozoa and as an inducer of acrosomal exocytosis (the acrosome reaction) (Howarth, 1992). One of the glycoprotein components of the inner perivitelline layer has significant sequence similarity to the ZPC family of proteins (Waclawek et al., 1998; Takeuchi, et al., 1999). Each successful sperm–inner perivitelline layer interaction is visible as a small hole in the inner perivitelline layer. These holes are observed both in vivo and in vitro (Steele et al., 1994) when intact ova (Bakst and Howarth, 1977) or sheets of inner perivitelline layer from either laid eggs or follicular oocytes are incubated with spermatozoa (Koyanagi et al., 1988; Bramwell and Howarth, 1992a; Steele et al., 1994; Robertson, 1997a). Molecules capable of competing with inner perivitelline layer-binding sperm ligands are thus likely to reduce the number of sperm–egg interactions, although it is not possible to determine whether inhibition occurs at the initial binding, at induction of the acrosome reaction or at secondary binding.

Preliminary investigations by Howarth (1990) demonstrated that preincubation of spermatozoa with solubilized inner perivitelline layer extracts before co-incubation with the inner perivitelline layer resulted in a significant reduction in the number of sperm–inner perivitelline layer interactions and that this inhibitory capacity was blocked by removal of the carbohydrate portion of the extracts (Howarth, 1992). These data were interpreted as evidence that the inner perivitelline layer sperm ligand is a carbohydrate. However, preincubation of chicken spermatozoa with inner perivitelline layer extracts induces an acrosome reaction within 5 min (Robertson and Wishart, 1996; Robertson et al., 1997b), thus generating a population of spermatozoa with mixed acrosomal status. Acrosome-reacted spermatozoa may not be able to initiate inner perivitelline layer binding, as has been demonstrated in mice (Sailing and Storey, 1979; Bleil et al., 1988) and pigs (Fazeli...
et al., 1997), or may be the only form of spermatozoa that can initiate inner perivitelline layer binding, as demonstrated in guinea-pigs (Huang et al., 1981). Such effects would introduce inherent difficulties in any attempt to identify the ligands involved in the initial stages of the sperm–inner perivitelline layer interaction. In the present study, these problems were circumvented by pretreating chicken inner perivitelline layer with lectins and glycosidases and assessing the level of sperm–inner perivitelline layer interaction on the intact but modified layer. The aim was to determine the role of oligosaccharides expressed on the egg coat in sperm binding, specifically N-linked oligosaccharides with terminal N-acetyl-d-glucosamine.

Materials and Methods

Materials

All chemicals were obtained from the Sigma Chemical Company Ltd, Poole, except PNGase F, endo-α-N-acetyl-galactosaminidase, fucosidase and sialidase, which were obtained from Oxford Glycosystems, Abingdon.

Birds and bird maintenance

ISA ‘Grandparent’ cockerels, Gallus domesticus, were obtained from ISA Poultry Services, Peterborough; female chickens were ISA Brown commercial layers. All birds were caged individually, under a photoperiod of 14 h light:10 h dark and fed a commercial breeder’s ration ad libitum.

Semen collection and preparation

Semen was collected from chickens by abdominal massage as described by Burrows and Quinn (1937). For most experiments, pooled ejaculates from three to four males were used to provide each sample. Each sample was diluted 1:4 in 0.15 mol NaCl l–1 with 20 mmol NaCl l–1 containing 1% (w / v) NaCl and the two layers were pulled apart using forceps. Isolated inner perivitelline layer was stored at 5°C in 1% (w / v) NaCl for up to 24 h.

Preparation of perivitelline layers from laid eggs

Inner and outer perivitelline layers were separated by acid treatment as described by Kido and Doi (1988). Fresh unfertilized eggs were cracked open and the yolks were washed in 1% (w / v) NaCl and rinsed in distilled water to remove the adhering albumen. Yolks were immersed in 75 ml of 0.01 mol HCl l–1 and incubated at 37°C for 1 h. After incubation, the yolk was punctured and the perivitelline layer removed and washed in 1% (w / v) NaCl until all adhering yolk was removed. The complete perivitelline layer was spread out in a Petri dish containing 1% (w / v) NaCl and the two layers were pulled apart using forceps. Isolated inner perivitelline layer was stored at 5°C in 1% (w / v) NaCl for up to 24 h.

Lectin binding to inner perivitelline layer

Stock solutions of 1 mg ml–1 were prepared in NaCl–TES for all lectins used, except for Canavalia ensiformis (Con A), which was prepared in NaCl–TES containing 0.1 mmol CaCl l–1 and 0.1 mmol MnCl l–1.

Pieces of inner perivitelline layer (approximately 0.5 cm × 0.5 cm) from laid chicken eggs were added to 1 ml lectin, diluted to a final concentration of 10 µg ml–1 in NaCl–TES fluorescein isothiocyanate (FITC)-labelled lectins and unlabelled lectins) or 100 µg ml–1 in NaCl–TES (unlabelled lectins only). Vials were incubated at 22°C (FITC-labelled lectins in a light-shielded container) for 30 min (FITC-labelled lectins), or for 4 h (unlabelled lectins) and the inner perivitelline layer was washed several times in NaCl–TES to remove unbound material. Labelled inner perivitelline layer was spread on a microscope slide and examined by epifluorescence at ×400 and ×1000 magnification.

The degree of fluorescence was scored using an arbitrary scale: highly fluorescent inner perivitelline layer = 3 (Fig. 1a); intermediate fluorescence = 2 (Fig. 1b); and marginal fluorescence = 1 (discernible only under low magnification). Inner perivitelline layer pieces treated with unlabelled lectin were incubated in the in vitro sperm–inner perivitelline layer assay.

Sperm–inner perivitelline interaction in vitro

Pieces of inner perivitelline layer approximately 0.5 cm × 0.5 cm were incubated at 40°C in 1 ml minimal essential medium with 1.25 × 107 spermatozoa for 5 min (Steele et al., 1994; Robertson et al., 1997a). After incubation, the pieces of inner perivitelline layer were washed in NaCl–TES to remove loosely adhering spermatozoa and spread on a microscope slide. These samples were examined at ×100 using dark ground optics and were recorded as video prints of three fields of view (each 0.55 mm²). The number of holes per mm² was calculated. Data were analysed using one-way ANOVA with Dunnett’s multiple comparison tests as appropriate (Robertson et al., 1997a).

Enzymatic cleavage of O- and N-linked glycans from follicular inner perivitelline layer

Follicular oocytes were removed from the ovaries of birds that had been killed by injection of 3 ml Euthatal (RBM Animal Health Ltd, Dagenham) into the wing vein. The abdomen was opened and the two largest follicular oocytes were removed from the ovaries. Follicles were cut open and washed in 1% (w / v) NaCl to remove adhering yolk. The inner perivitelline layer was teased gently from the follicle using forceps, washed thoroughly in several changes of 1% (w / v) NaCl and rinsed in distilled water to remove the granulosa cells. Isolated inner perivitelline layer was stored in NaCl–TES at 5°C for up to 24 h.

N-linked glycans were removed selectively by incubating individual pieces of follicular inner perivitelline layer of approximately 1.5 cm × 1 cm in 18 mU peptide-N-glycosi-
dase F (PNGase F) ml⁻¹ in 300 μl sodium phosphate buffer (20 mmol l⁻¹ at pH 7.5) containing 50 mmol EDTA l⁻¹, 1 mmol benzamidine l⁻¹, 10 μg trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64) ml⁻¹, 10 μg phenylmethylsulfonyl fluoride (PMSF) ml⁻¹ and 10 μg pepstatin A ml⁻¹.

Removal of O-linked glycans was carried out by incubating individual pieces of follicular inner perivitelline layer of approximately 1.5 cm × 1 cm, with 12 U endo-α-N-acetylgalactosaminidase ml⁻¹ in 300 μl of 100 mmol sodium citrate buffer l⁻¹ pH 6.0 containing 100 μg BSA, 0.02% (w/v) sodium azide, 1 mmol benzamidine l⁻¹, 10 μg trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64) ml⁻¹, 10 μg phenylmethylsulphonyl fluoride (PMSF) ml⁻¹, 10 μg pepstatin A ml⁻¹ and 10 mmol galactoactone l⁻¹. Fucosidase at 0.7 U ml⁻¹ and 0.7 U sialidase ml⁻¹ were added to the reaction mixture to remove any fucose or sialic acid attached to the core disaccharide that would prevent hydrolysis of the GalNAc–peptide linkage (Piller and Piller, 1993). Incubation supernatants were assayed for sugars using an orcinol total sugar assay (White and Kennedy, 1994). Controls were individual pieces of inner perivitelline layer incubated in each type of buffer without the addition of enzymes.

**Results**

**Lectin binding to inner perivitelline layer**

Inner perivitelline layer isolated from laid eggs was incubated with a variety of FITC-labelled lectins and the efficiency of binding was determined by comparing the intensity of fluorescence on a subjective scale (Fig. 1, Table 1). WGA (Triticum vulgarius) and succinyl-WGA (S-WGA) both stained the inner perivitelline layer fibres intensely, despite small differences in binding specificity. Con A (Canavalia ensiformis), PNA (Arachis hypogea), PSA (Pisum sativum), STA (Tetragonolobus purpureas) and UEA II (Ulex europaeus II) showed moderate staining and STA (Solanus tuberosum) weak staining, which was only detectable under low magnification. There was no perceptible difference in the intensity of lectin staining of inner perivitelline layer removed from the germinal disc region compared with other regions, indicating that lectin-binding sites were equally distributed throughout the inner perivitelline layer.

The effect of lectin pretreatment on sperm–egg interaction was assessed using the *in vitro* sperm–inner perivitelline layer interaction assay; the number of holes in the inner perivitelline layer was indicative of the number of sperm–egg interactions. Pretreatment with WGA and S-WGA completely inhibited hydrolysis of the inner perivitelline layer by spermatozoa (*P* < 0.01) at both 10 and 100 μg lectin ml⁻¹ (Table 1). At the higher concentration, pretreatment with STA, UEA II and LTA reduced the number of holes formed in the inner perivitelline layer by approximately 40% (compared with the control), whereas pretreatment with Con A and PNA reduced hydrolysis by 30% (all *P* < 0.01) and PSA by 25% (*P* < 0.05). At the lower concentration none of the lectins significantly reduced sperm hydrolysis. Inhibition by WGA was significantly (*P* < 0.001) blocked by preincubating the lectin with 0.2 mol N,N’-diacetylchitobiose l⁻¹ (Table 2). When the concentration of N,N’-diacetylchitobiose was reduced to 0.04 mol l⁻¹ the pretreated WGA only reduced hydrolysis to 40% of the control values.

**Effect of saccharides on sperm–inner perivitelline layer interactions in vitro**

A range of monosaccharides and the polysaccharide fucoidin were tested as potential inhibitors of sperm–inner perivitelline layer interaction by incorporation into the *in vitro* interaction assay. Sperm motility was not affected at the concentrations of saccharide used (1.0 mg fucoidin ml⁻¹, 0.075 mol N-acetyl-d-glucosamine l⁻¹, 0.1 mol d-glucose l⁻¹, 0.1 mol d-galactose l⁻¹, 0.1 mol d-fucose l⁻¹ and 0.1 mol l-fucose l⁻¹). After the addition of d-manose to the assay, a small but significant increase was seen in the number of holes in the inner perivitelline layer (*P* < 0.01). Only fucoidin and d-GlcNAc inhibited sperm hydrolysis of the inner
Table 1. Affinity of the inner perivitelline layer from laid eggs for fluorescein isothiocyanate (FITC)-labelled lectins and the effect of preincubation with lectins on sperm–egg interaction in chickens

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Carbohydrate affinity(^a)</th>
<th>Intensity of fluorescence staining(^b)</th>
<th>Inner perivitelline layer holes (percentage of controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100 μg lectin ml(^{-1}) ((n = 8))</td>
<td>10 μg lectin ml(^{-1}) ((n = 6))</td>
</tr>
<tr>
<td>Triticum vulgaris (WGA)</td>
<td>GlcNAc(β1,4GcNAc)(_{1–2}) &gt; βGcNAc &gt; NeuNAc</td>
<td>3</td>
<td>0.00 ± 0</td>
</tr>
<tr>
<td>Succinyl Triticum vulgaris (S-WGA)</td>
<td>GlcNAc(β1,4GcNAc)(_{1–2}) &gt; βGlcNAc</td>
<td>2</td>
<td>0.00 ± 0</td>
</tr>
<tr>
<td>Canavalia ensiformis (Con A)</td>
<td>α-Man &gt; α-β-Glc &gt; α-GlcNAc</td>
<td>2</td>
<td>73.19 ± 6.8</td>
</tr>
<tr>
<td>Arachis hypogea (PNA)</td>
<td>α-Man &gt; α-Glc = αGlcNAc</td>
<td>2</td>
<td>70.10 ± 6.37</td>
</tr>
<tr>
<td>Ulex europaeus II (UEA II)</td>
<td>L-Fucα1,2Galβ1,4GcNAc &gt; GlcNAc(β1-4GlcNAc)(_{1–3})</td>
<td>2</td>
<td>52.75 ± 7.05</td>
</tr>
<tr>
<td>Solanum tuberosum (STA)</td>
<td>GlcNAc(β1,4GcNAc)(_{1–4})</td>
<td>1</td>
<td>61.94 ± 17.71</td>
</tr>
<tr>
<td>Tetragonolobus purpureas (LTA)</td>
<td>α-1-Fuc &gt; 1-Fuc α1,2Galβ1,4GalNAc &gt; Fucα1,2Galβ1,3GalNAc</td>
<td>2</td>
<td>66.53 ± 11.01</td>
</tr>
<tr>
<td>Pisum sativum (PSA)</td>
<td>α-Man &gt; α-Glc = α-GlcNAc</td>
<td>2</td>
<td>76.70 ± 7.57</td>
</tr>
</tbody>
</table>

\(^a\) Lectin specificity according to Goldstein and Poretz (1986).
\(^b\) FITC-labelled lectin (10 μg ml\(^{-1}\)) \((n = 3)\); 3 = strong, 1 = weak.
ND: no data available.
perivitelline layer (P < 0.01): fucoidin reduced sperm hydrolysis by less than 25%, whereas N-acetyl-d-glucosamine reduced hydrolysis by more than 70% (Table 3), indicating that the inner perivitelline layer sperm-binding moiety contains N-acetyl-d-glucosamine and possibly a fucose polymer.

Effect of endoglycosidase treatment on sperm–inner perivitelline layer interaction in vitro

When pieces of follicular inner perivitelline layer were incubated with either N-glycosidase or O-glycosidases of equivalent activities, PNGaseF released approximately 34.99 ± 8.1 μg carbohydrate per 1.5 cm² inner perivitelline layer sheet and O-glycosidase treatment released 25.53 ± 8.1 μg per inner perivitelline layer sheet. No significant difference (99.72 ± 14.64%) of untreated control inner perivitelline layer holes; two samples unpaired t test; P > 0.5) was observed in the ability of spermatozoa to hydrolyse O-glycosidase-treated inner perivitelline layer sheets and control inner perivitelline layer sheets incubated in buffer alone. Inner perivitelline layer treated with PNGaseF showed a reduction of 86% in sensitivity to hydrolysis by spermatozoa in comparison with the control (15.15 ± 4.83% of untreated control inner perivitelline layer holes; unpaired t test; P < 0.0001), indicating that the interaction of spermatozoa and inner perivitelline layer is dependent on N-linked glycans.

**Table 2. Effect of WGA (Triticum vulgarius) and WGA pretreated with N,N-diacytelchitobiose on the ability of chicken spermatozoa to hydrolyse the inner perivitelline layer**

<table>
<thead>
<tr>
<th>WGA (μg ml⁻¹)</th>
<th>N,N-diacytelchitobiose (mol l⁻¹)</th>
<th>Inner perivitelline layer holes (percentage of controls, n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>0.00</td>
<td>–</td>
</tr>
<tr>
<td>100</td>
<td>0.04</td>
<td>40.10 ± 5.2</td>
</tr>
<tr>
<td>100</td>
<td>0.20</td>
<td>89.87 ± 8.7</td>
</tr>
</tbody>
</table>

*WGA was preincubated with N,N diacytelchitobiose for 30 min and incubated with the inner perivitelline layer.

**Table 3. The effect of saccharide competition of sperm–inner perivitelline layer interaction**

<table>
<thead>
<tr>
<th>Saccharide</th>
<th>Concentration</th>
<th>Inner perivitelline layer holes (percentage of controls, n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose (D-Glc)</td>
<td>0.1 mol l⁻¹</td>
<td>91.1 ± 7.85</td>
</tr>
<tr>
<td>D-galactose (D-Gal)</td>
<td>0.1 mol l⁻¹</td>
<td>95.33 ± 10.33</td>
</tr>
<tr>
<td>D-fucose (D-Fuc)</td>
<td>0.1 mol l⁻¹</td>
<td>93.79 ± 5.65</td>
</tr>
<tr>
<td>L-fucose (L-Fuc)</td>
<td>0.1 mol l⁻¹</td>
<td>86.35 ± 16.41</td>
</tr>
<tr>
<td>D-mannose (D-Man)</td>
<td>0.1 mol l⁻¹</td>
<td>113.78 ± 12.46</td>
</tr>
<tr>
<td>N-acetyl-d-glucosamine (D-GlcNAc)</td>
<td>0.075 mol l⁻¹</td>
<td>26.63 ± 5.52</td>
</tr>
<tr>
<td>Fucoidin</td>
<td>1.0 mg ml⁻¹</td>
<td>76.93 ± 6.11</td>
</tr>
</tbody>
</table>

**Discussion**

The current study demonstrates that carbohydrates play a crucial role in avian sperm–egg interactions, as tested in vitro using an assay that required spermatozoa to bind to the inner perivitelline layer sheet, undergo an acrosome reaction and hydrolyse a small hole. Since it has been established that hydrolysis of the inner perivitelline layer by acrosin is unaffected by WGA, inhibition of this interaction after modification of the inner perivitelline layer with enzymes or lectins could thus be a consequence of reduced binding of spermatozoa, reduced ability to induce the acrosome reaction or a combination of both. The results of the present study demonstrate that carbohydrates play an essential role in modulating one or both of these processes.

Glycosidase pretreatment showed that the removal of N-linked but not O-linked sugars could inhibit the avian sperm–inner perivitelline layer interaction. In mammals, extensive studies of mouse sperm–egg interactions have led to the general assumption that the sperm-binding activity of the zona pellucida is associated with O-linked oligosaccharides located near the C-terminus of mZP3, a ZPC type glycoprotein (Florman and Wasserman, 1985; Rosiere and Wasserman, 1992; Kinloch et al., 1995; Litscher and Wasserman, 1996; Chen et al., 1998). However, in pigs there is evidence for the involvement of both O-linked and N-linked carbohydrates in sperm binding (Yurewicz et al., 1991; Yonezawa et al., 1995) and in sea urchins N-linked carbohydrates were found to induce the acrosome reaction (Keller and Vacquier, 1994), although O-linked oligosaccharides bound acrosome-reacted spermatozoa before penetration (Dhume and Lennarz, 1995). Thus, although one of the inner perivitelline layer glycoproteins appears to belong to the ZPC family, sperm–inner perivitelline layer interactions in birds, as distinct from sperm–zona interaction in mammals, is mediated by N-linked oligosaccharides with no apparent requirement for O-linked oligosaccharides.

Lectin pretreatment showed that although the inner perivitelline layer proteins bound a range of lectins, only those lectins with affinity for N-acetyl-d-glucosamine monomers (WGA and S-WGA) were able to inhibit sperm–egg interaction completely. There are two explanations for the inability of spermatozoa to hydrolyse the inner perivitelline layer after lectin treatment: (i) lectins bind to the inner perivitelline layer oligosaccharides and mask spatially adjacent inner perivitelline layer-associated sperm binding factors; or (ii) lectins bind to and thus mask specific oligosaccharides that have an important role in sperm binding. The limited inhibition of sperm–inner perivitelline layer interactions exerted by high concentrations of all lectins tested would indicate that this inhibition may be a consequence of steric hindrance. However, the finding that WGA and S-WGA exerted total inhibition of sperm–egg interaction at low concentration indicates that these lectins
may block specific sperm binding sites on inner perivitelline layer glycoproteins. In order of preference, WGA and S-WGA bind to the diacycthibiose core of N-linked glycans and N-acetyl-D-glucosamine, and WGA alone binds to sialic acid. STA and UEII, which do not exert total inhibition of sperm–egg interaction, also recognize D-GlcNAC residues but only in the form of β(1,4)-linked oligomers. Thus, there is evidence that strong inhibition of sperm–inner perivitelline layer interaction has been achieved by masking a terminal N-acetyl-D-glucosamine. Further evidence that sperm–inner perivitelline layer interactions are mediated by specific carbohydrates has been provided by the ability of monosaccharides to interfere with this process. Although mannose inexplicitly had a positive effect on sperm–inner perivitelline layer interactions, two saccharides inhibited this process significantly. Fucoidin, which inhibited sperm–inner perivitelline layer interactions at high concentrations, also inhibited sperm–zona interaction in rats (Shalgi et al., 1986), guinea-pigs (Huang et al., 1982) and humans (Oehninger et al., 1990). However, the fucose-binding lectins (UEII and LTA) at low concentrations and 1-fucose monosaccharides did not inhibit sperm–inner perivitelline layer interactions, indicating that a terminal fucose is unlikely to be involved in sperm binding. N-acetyl-D-glucosamine monosaccharides significantly inhibited sperm–egg interactions and in combination with the lectin binding data, this finding provides evidence that terminal N-acetyl-D-glucosamine is important in mediating sperm–egg interaction in birds.

The authors thank the Biotechnology and Biological Sciences Research Council and the University of Abertay Dundee for funding this work.

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