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# Lectin histochemical localization of N- and O-linked oligosaccharides during the spermiogenesis of the urodele amphibian *Pleurodeles waltl*

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**The aim of this work is the characterization of the glycoconjugates of the spermatids during the spermiogenesis of the testis of an urodele amphibian, *Pleurodeles waltl*, by means of lectins in combination with several chemical and enzymatic procedures, in order to establish the distribution of N- and O-linked oligosaccharides in these cells. The acrosome was the most relevant lectin-labeled structure. The O-linked oligosaccharides contained DBA- and SBA-positive GalNAc, AAA-positive Fuc and PNA-positive Galβ1,3GalNAc. Sialic acid was scarcely observed, the Neu5Acα2,-3Galβ1,4GlcNAc sequence was found in N-linked oligosaccharides. Additionally, N-linked oligosaccharides containing HPA-positive GalNAc and AAA-positive Fuc were found. Moreover, with some lectins the acrosome showed a variable composition of the oligosaccharides in the different steps of the sperm maturation. Some residues were found only in the early steps in maturating acrosome, while others were in the later steps, showing that acrosomal glycoconjugates are modified during acrosome development in spermiogenesis. The changes observed during acrosome maturation suggest the existence of a predetermined pattern of storage of the acrosome components and a progressive compression of them.**

***Keywords:*** **lectin histochemistry, amphibian, testis, spermiogenesis, acrosome**

## Introduction

A role for the oligosaccharides in the control of the spermiogenesis and the acrosomal development [1–3] and during the fertilization [4–6] has been suggested. Thus, the oligosaccharide composition of mammal testis [7–12] and less-extensely, non-mammal testis [13–15] has been investigated.

Oligosaccharide chains of glycoproteins have been classified into two families. The so-called N-linked oligosaccharides are those in which an N-glycosidic bond is established between N-acetylglucosamine (GlcNAc) and the amide nitrogen of asparagine [16]. The second group, the named O-linked oligosaccharides, comprises the sugar chains linked to hydroxylated amino acids of proteins (serine and/or threonine). In this second family, the sugar that binds the polypeptide is N-acetylgalactosamine (GalNAc) in mucins, xylose in proteoglycans, galactose (Gal) in collagen, and GlcNAc in nuclear pores and cytoplasmic proteins [17].

Several morphological techniques give direct information about the topographic distribution of sugar residues of the oligosaccharides [18]. Some of them are based on the use of lectins, which are proteins or glycoproteins of non-immune origin which bind specifically to carbohydrate groups [18]. This methodology is useful because lectin-sugar binding is not species-specific. Moreover, in combination with enzymatic and chemical deglycosylation, lectins give information on the N- or O-linked nature of oligosaccharides [19–21].

The application of lectin histochemistry to the mammalian testes has disclosed the distribution of N- and O-linked oligosaccharides in the spermatogenetic cells [22,23], in the acrosome [2], as well as the sequential glycosylation processes of the acrosomal development [3]. However, in lower vertebrates, studies are scarce and the detection of carbohy-

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drates has been carried out by means of conventional histochemical techniques [13,24], and rarely by means of lectin histochemistry [14,15]. No report exists about lectin histochemistry of the urodele testis.

The testes of most of urodeles are composed of two to four lobes with identical organization and synchronic maturation. Each lobe consists of multiple spherical lobules connected to a branched system of ductuli efferentes. Most of each lobule comprises a single germ cell type. Germ cell development in the lobules progresses from the anterior to the posterior testicular poles [25]. This testicular organization makes the urodele testis a useful tool for the study of the spermatogenesis in vertebrates. Moreover, the peculiar structure of the urodele spermatozoon and their spermiogenic process make this an interesting model of cell differentiation, at both the ultrastructural and the functional levels [26].

During spermiogenesis, the round spermatids, which have a spherical nucleus, a round pro-acrosomal granule near the nucleus and a ciliary structure, gradually become elongated [25–28]. In the elongated spermatids, a forming neckpiece is observed where the tail is formed at the opposite end to the pro-acrosomal granule. Finally, the mature spermatozoa have a very elongated head, with a nucleus covered on this tip by a small thin acrosome, and the neckpiece at the opposite side to the acrosome surrounding the initium of the tail. The tail is a complex structure formed by a microtubular axoneme and an axial rod connected by an undulating membrane [25,26].

The aim of this work was the characterization of glycoconjugates of spermatids during the spermiogenesis of the testis of an urodele amphibian, *Pleurodeles waltl*, by means of 14 different lectins in combination with several chemical and enzymatic procedures, in order to establish the distribution of N- and O-linked oligosaccharides in these cells. We discuss these results in relation to those obtained in the testis of other species, especially in mammals. The present results contribute to the knowledge of the modification of the glycoconjugates during spermatozoon development, including the acrosome and other subcellular structures.

## Material and methods

### Tissue preparation

Fourteen male adult specimens of Spanish newt, *Pleurodeles waltl*, were studied. They were maintained in our laboratory under standard conditions. The animals were anaesthetized with 3-aminobenzoic acid ethyl ester (MS-222, Sigma Química, Alcobendas, Madrid, Spain), and perfused with Bouin's solution. After a few minutes, the testes were removed and immersed in the same fixative for 12 hours. After washing in 30% (v/v) ethanol to remove the excess of fixative, the samples were dehydrated in ethanol, embedded in paraffin and 5 μm-thick sections were obtained.

### Lectin histochemistry

The present study has been made using lectins that cover the entire spectrum of carbohydrate constituents of glycoproteins, i.e., lectins that bind fucose (Fuc), GalNAc, Gal, sialic acid (Neu5Ac), GlcNAc and mannose (Man). The carbohydrate binding specificity of the lectins used in this work is summarized in Table 1.

Histochemical lectin labeling was performed using horseradish peroxidase (HRP)- and digoxigenin (DIG)-conjugated lectins. Lectin binding patterns were established on paraffin

**Table 1.** Carbohydrate binding specificity of lectins [18,32]

| *Lectin* | *Abbreviation* | *Carbohydrate binding specificity* |
|---|---|---|
| *Glycine max* (soybean) agglutinin | SBA | GalNAc |
| *Dolichos biflorus* agglutinin | DBA | GalNAc |
| *Helix pomatia* agglutinin | HPA | GalNAc > Gal |
| *Lotus tetragonolobus* agglutinin | LTA | Fuc |
| *Ulex europaeus* agglutinin-I | UEA-I | Fuc |
| *Aleuria aurantia* agglutinin | AAA[a] | Fuc |
| *Arachis hypogea* (peanut) agglutinin | PNA | Gal $\beta$1,3 GalNAc |
| *Datura stramonium* agglutinin | DSA | Gal $\beta$1,4 GlcNAc |
| *Limax flavus* agglutinin | LFA | Neu5Ac |
| *Maackia amurensis* agglutinin | MAA | Neu5Ac $\alpha$2,3 Gal $\beta$1,4 GlcNAc |
| *Sambucus nigra* agglutinin | SNA | Neu5Ac $\alpha$2,6 Gal/GalNAc |
| *Triticum vulgaris* (wheat germ) agglutinin | WGA | $(GlcNAc)_n$ > Neu5Ac |
| *Canavalia ensiformis* agglutinin (Concanavalin A) | ConA | Man > Glc |
| *Galanthus nivalis* agglutinin | GNA | $(Man)_n$ |

[a] The acronym AAA is used for two lectins, those of *Aleuria aurantia* and *Anguilla anguilla*. In this work AAA is employed for the *Aleuria aurantia* agglutinin; *Anguilla anguilla* agglutinin was not used.

sections without previous treatment, after chemical deglycosylation ($\beta$-elimination, which removes O-linked oligosaccharides) and after enzymatic deglycosylation with Endoglycosidase F/Peptide N-glycosidase F (to remove N-linked oligosaccharides). For wheat germ agglutinin (WGA), which labels both sialic acid and GlcNAc, the same three treatments were repeated after acid hydrolysis. After this procedure staining was due to GlcNAc. For *Canavalia ensiformis* agglutinin (ConA), which recognizes both Man and glucose (Glc), the three treatments were done again after glucose-oxidase treatment. After this, which converts glucose (Glc) into gluconic acid, staining was due to Man.

The following cell types were analyzed [25,27,28]: round spermatids, elongated spermatids and sperm bundles. Moreover, some subcellular structures were also analyzed: 1) labeling in cell cytoplasm; 2) proacrosomal granules in round and elongated spermatids and acrosome in spermatozoa; and 3) tail in round and elongated spermatids and sperm.

In order to know the relative abundance of sugars in the cells and cellular structures labeled, the staining intensity was studied. Cells and cellular structures were arbitrarily classified into four categories according to their labeling intensity: no labeling (−), weak (+), moderate (++) and strong (+++).

Histochemical staining using HRP-conjugated lectins was performed as previously reported [29]. Briefly, endogenous peroxidase was blocked with 0.3% (v/v) hydrogen peroxide in Tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.5). Then, the sections were incubated for 2 h at room temperature with the following HRP-conjugated lectins: Concanavalin A (ConA, 20 μg/ml), peanut agglutinin (PNA, 50 μg/ml), wheat germ agglutinin (WGA, 10 μg/ml), *Helix pomatia* agglutinin (HPA, 6 μg/ml), *Dolichos biflorus* agglutinin (DBA, 30 μg/ml), soybean agglutinin (SBA, 18 μg/ml), *Lotus tetragonolobus* agglutinin (LTA, 50 μg/ml), *Ulex europaeus* agglutinin (UEA-I, 20 μg/ml) from Sigma Química (Alcobendas, Madrid, Spain), and *Limax flavus* agglutinin (LFA, 25 μg/ml) from EY (San Mateo, California). Peroxidase was developed with 0.05% (v/v) 3,3′-diaminobenzidine and 0.015% (v/v) hydrogen peroxide in TBS. Eventually, sections were counterstained with hematoxylin.

Histochemical staining using DIG-labeled lectins was performed as previously reported [2,30]. Briefly, after endogenous peroxidase blocking with 0.3% (v/v) hydrogen peroxide in TBS, the sections were incubated with 1% (v/v) BSA in TBS for 10 min and the sections were incubated for 1.5 h at room temperature with the following digoxigenin-conjugated lectins (Boehringer Manheim, Barcelona, Spain): *Galanthus nivalis* agglutinin (GNA, 60 μg/ml), *Datura stramonium* agglutinin (DSA, 10 μg/ml), *Sambucus nigra* agglutinin (SNA, 30 μg/ml), *Maackia amurensis* agglutinin (MAA, 10 μg/ml) and *Aleuria aurantia* agglutinin (AAA, 20 μg/ml). After washing, sections were incubated with HRP-conjugated anti-DIG-antibodies (Boehringer Mannheim Biochemica, Barcelona, Spain) for 1 h at 0.6 U/ml in TBS supplemented with 1% BSA. Peroxidase was developed as described and the sections were counterstained with hematoxylin.

### Chemical deglycosylations ($\beta$-elimination and acid hydrolysis)

To eliminate O-linked oligosaccharides, paraffin sections were treated with 0.5 N sodium hydroxide in 70% (v/v) ethanol at 4°C for 10 days according to Ono et al. (19).

Acid hydrolysis, which eliminates sialic acid residues [31,32] was performed by immersing the sections in 0.1 M hydrochloric acid for 2–3 hr at 82°C.

### Enzymatic deglycosylation (Peptide N-glycosidase F treatment)

To remove N-linked oligosaccharides, paraffin sections were incubated in the buffer (0.1 M Tris, 150 mM NaCl, 2.5 mM EDTA, pH 9) containing 1% (v/v) Triton X-100 for 1 h and then in the same buffer with 1% (v/v) BSA for 10 min. After brief washing in the buffer without BSA, the sections were incubated in the enzyme Endoglycosidase F/Peptide N-glycosidase F (Endo F/PNGase F, Boehringer Mannheim Biochemica, Barcelona, Spain) at 6 U/ml for 3 days. At pH 9, PNGase-F activity predominated over Endo F activity so that cleavage of $\beta$-aspartyl glycosyl linkage (GlcNAc $\beta$1-N Asn) was preferred [21].

### Carbohydrate residues conversion (glucose-oxidase treatment)

To convert Glc into gluconic acid, the paraffin sections were first washed in sodium acetate buffer at pH 5.0. Then, they were incubated over night with 200 U/ml Type VII glucose-oxidase from *Aspergillus niger* (Sigma, Alcobendas, Madrid, Spain) at 37°C in a moist chamber. Finally, sections were washed in TBS three times for 5 min each and were stained with ConA lectin as usual.

### Controls

The following controls were used: 1) substitution of the lectins and antibodies by the corresponding buffer, 2) preincubation of the lectins with the corresponding hapten sugar inhibitor (Fuc for AAA, UEA-I and LTA; GlcNAc for WGA; GalNAc for HPA, SBA and DBA; α-methyl-mannopyranoside for ConA and GNA; Neu5Ac, for LFA; lactosamine for DSA; Gal for PNA; α2,3sialyllactosamine for MAA and α2,6sialyllactoctosamine for SNA; Sigma, Spain) at a concentration of 0.2 M; and 3) preabsorption of the antibodies with the corresponding antigen.

## Results

The results are summarized in Table 2. The control sections were negative. Here comment is made only on the most interesting findings.

**Table 2.** Lectin labeling pattern in the mature part of the testis of *Pleurodeles waltl*. C: cytoplasmic labeling, A: proacrosomal granule and acrosome, T: tail. Staining intensity: − negative, + weak, ++ moderate, +++ strong. When two symbols are employed, for example −/+, it indicates some variability

| | *Round spermatids* | | | *Elongated spermatids* | | | *Spermatozoa bundles* | |
|---|---|---|---|---|---|---|---|---|
| | *C* | *A* | *T* | *C* | *A* | *T* | *A* | *T* |
| **GalNAc-binding lectins** | | | | | | | | |
| SBA | − | ++ | ++ | − | +++ | ++ | ++ | + |
| *β*-elimination | − | − | ++ | − | − | ++ | − | + |
| PNGase-F | − | ++ | ++ | − | +++ | ++ | ++ | + |
| DBA | − | +++ | − | − | +++ | + | +++ | +[a] |
| *β*-elimination | − | − | − | − | − | + | +++ | + |
| PNGase-F | − | +++ | − | − | +++ | + | +++ | +[a] |
| HPA | − | +++ | − | − | +++ | − | +++ | − |
| *β*-elimination | − | ++ | − | − | ++ | − | ++ | − |
| PNGase-F | + | +++ | ++ | + | +++ | ++ | +++ | ++ |
| **Fuc-binding lectins** | | | | | | | | |
| LTA | − | −/++ | − | +/++ | − | − | − | − |
| *β*-elimination | − | − | − | − | − | − | − | − |
| PNGase-F | − | − | − | + | − | − | − | − |
| UEA-I | + | +++ | + | + | − | + | − | − |
| *β*-elimination | − | +++ | − | + | − | − | − | −[b] |
| PNGase-F | + | − | + | + | − | + | − | −[b] |
| AAA | + | +++ | + | + | +++ | + | ++ | ++ |
| *β*-elimination | + | ++ | + | ++ | + | ++ | −/+ | ++ |
| PNGase-F | + | +/++ | + | + | + | + | −/+ | ++ |
| **Gal-binding lectins** | | | | | | | | |
| PNA | + | +++ | ++ | ++ | +++ | +/++ | ++ | + |
| *β*-elimination | − | −/+ | − | − | −/+ | − | − | −/+ |
| PNGase-F | + | +++ | ++ | ++ | +++ | +/++ | ++ | + |
| DSA | + | − | ++ | ++ | − | ++ | − | ++ |
| *β*-elimination | + | − | ++ | ++ | − | ++ | − | ++ |
| PNGase-F | + | − | + | ++ | − | + | − | + |
| **Neu5Ac-binding lectins** | | | | | | | | |
| LFA | ++ | − | + | ++ | − | ++ | − | ++ |
| *β*-elimination | + | − | +/++ | +/++ | − | +/++ | − | +/++ |
| PNGase-F | −/++ | − | −/++ | −/++ | − | −/++ | − | −/++ |
| MAA | + | − | ++ | ++ | − | ++ | − | ++ |
| *β*-elimination | + | + | ++ | + | − | +/++ | − | + |
| PNGase-F | + | − | + | + | − | + | − | ++ |
| SNA | − | − | ++ | − | − | ++ | − | ++ |
| *β*-elimination | − | − | +++ | − | − | ++ | − | ++/+++ |
| PNGase-F | −/+ | − | − | − | − | + | − | + |
| **GlcNAc- and Neu5Ac-binding lectin** | | | | | | | | |
| WGA[c] | + | − | ++ | + | − | ++ | ++ | ++ |
| *β*-elimination | + | − | ++ | + | − | ++ | +/++ | ++ |
| PNGase-F | + | − | ++ | + | ++ | ++ | +++ | ++ |
| Acid hydrolysis+WGA | + | − | + | +[d] | −/++ | + | − | + |
| *β*-elimination | −/+ | − | ++ | −[d] | − | ++ | − | +/++ |
| PNGase-F | −/+ | − | ++ | + | ++ | ++ | −/++ | ++ |
| **Man-binding lectins** | | | | | | | | |
| ConA | ++ | − | − | ++ | − | − | + | +[e] |
| *β*-elimination | + | ++ | − | + | − | − | ++ | +[e] |
| PNGase-F | −/+ | +/++ | −/+ | −/+ | − | −/+ | − | − |
| Glc-oxidase+ConA | +/++ | − | − | +/++ | − | ++ | − | ++ |
| *β*-elimination | +/++ | − | − | +/++ | − | − | − | − |
| PNGase-F | +/++ | − | − | +/++ | − | + | − | − |
| GNA | + | ++ | − | +/++ | − | − | ++ | − |
| *β*-elimination | −/+ | ++ | − | +/++ | − | − | −/+ | − |
| PNGase-F | −/+ | − | − | −/+ | − | − | − | − |

[a]The neck piece is not labeled (−). [b]There is labeling (+) only in the neck piece. [c]WGA lectin binds to both Neu5Ac and GlcNAc residues. [d]The middle region of the late spermatid head shows a strong labeling (+++). [e]Only the intermediate piece of the tail is labeled, but not the principal piece.

N-acetylgalactosamine (GalNAc)

Usually, there was no spermatid cytoplasmic labeling with any of the three GalNAc-binding lectins used (Figs. 1 and 2), except with HPA-lectin after PNGase-F treatment.

The acrosome was positive for the three GalNAc-binding lectins (Figs. 1a and 2a). The labeling of SBA and DBA was not observed when the sections were pre-treated for $\beta$-elimination procedure (Fig. 1b), except for the spermatozoa acrosome, which was positive for DBA.

The tails of spermatids and spermatozoa were positive for SBA and DBA (Fig. 1a), except for the round spermatids which were not labeled by DBA. In contrast, HPA was only positive after PNGase-F pre-treatment (Fig. 2b). Occasionally, it was clearly observed that the neck piece of the tail in the spermatozoa was negative.

Fucose (Fuc)

LTA only labeled the acrosome of round spermatids and the cytoplasm of elongated spermatids.

The cytoplasm of cells showed a slight labeling with UEA-I and AAA. The UEA-I staining usually disappeared with $\beta$-elimination pre-treatment. The acrosome was positive for AAA (Fig. 3), while UEA-I only labeled the acrosome of round spermatids. The tails of the spermatozoa bundles were only positive for AAA.

Galactose (Gal)

The cytoplasm of cells was stained with both PNA and DSA. The acrosome was strongly stained with PNA (Fig. 4a), and the labeling almost disappeared with $\beta$-elimination (Fig 4b). The tail was positive for both lectins; however PNA staining was not observed after $\beta$-elimination procedure.

N-acetylneuraminic acid (Neu5Ac)

The cytoplasm of the both round and elongated spermatids was positive for LFA and MAA. The acrosome was negative with the three lectins employed, except for light staining for MAA after $\beta$-elimination in round spermatids. The tail of spermatids and spermatozoon were labeled by the three lectins (Fig. 5).

N-acetylglucosamine (GlcNAc)

Usually, WGA staining after acid hydrolysis followed the same pattern as normal WGA labeling, but the intensity of staining was lower.

The acrosome showed variable staining and in round spermatids it was negative. In elongated spermatids the PNGase-F treatment revealed WGA staining that remained after sialic acid removal with acid hydrolysis. The removal of sialic acid also revealed the presence of WGA-binding carbohydrates in the acrosome of elongated spermatids. The spermatozoa bundles were positive for WGA in the acrosome, but this staining disappeared with acid hydrolysis pre-treatment, except occasionally, for the sequence acid hydrolysis/PNGase-F/WGA.

Mannose (Man)

To elucidate if ConA labeling was Man-specific, we also previously carried out glucose-oxidase pre-treatment.

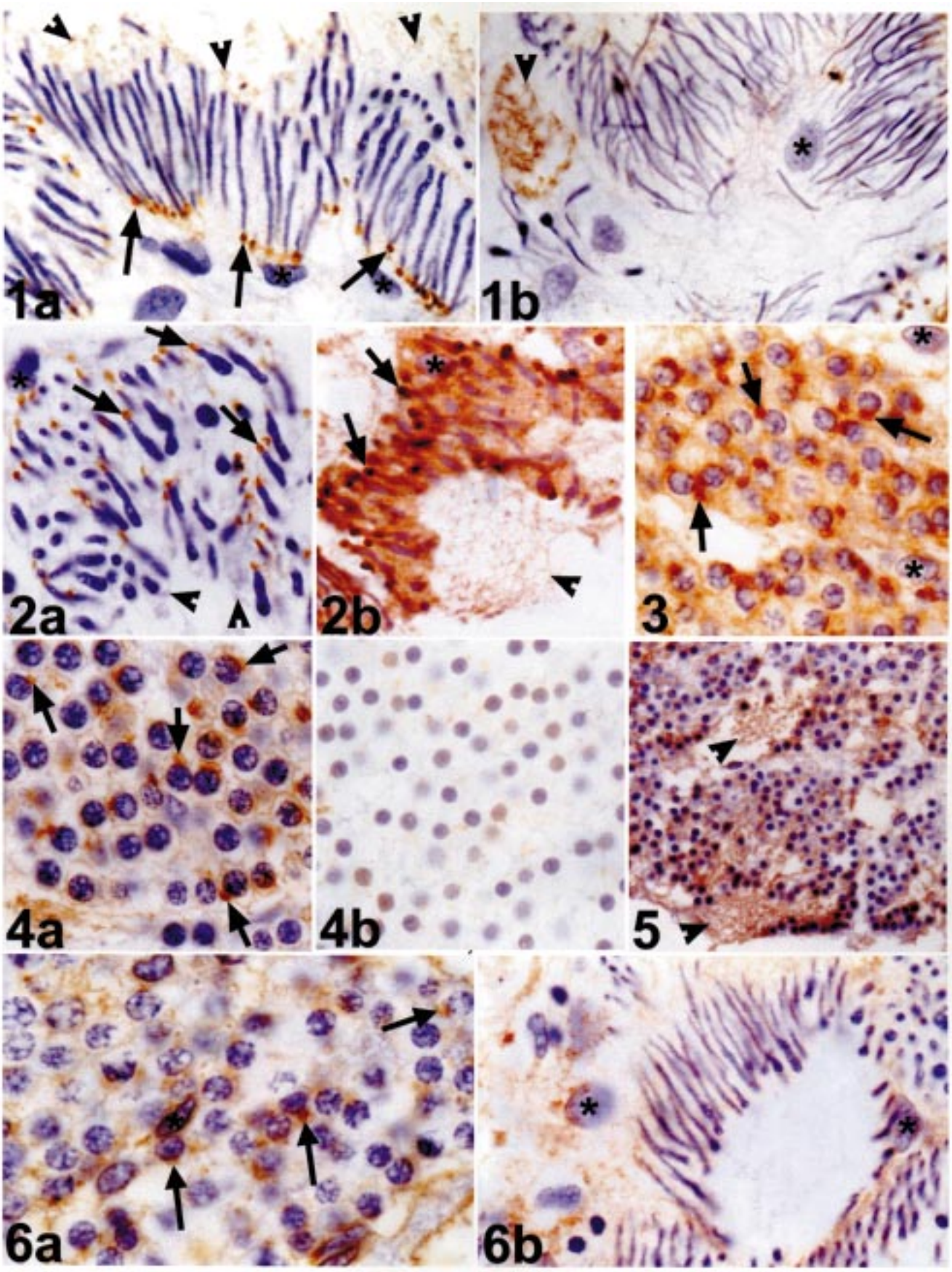
The cytoplasm of the cells was positive for both ConA and GNA. Glucose-oxidase pre-treatment usually decreased the ConA staining. The acrosome shows a variable staining (Fig. 6a and 6b): the elongated spermatids were negative, while in less- and more-mature stages, GNA lectin was positive. The ConA labeling of the acrosome disappeared with glucose-oxidase treatment.

## Discussion

In the present article, the glycan composition of oligosaccharides of the *Pleurodeles waltl* spermatids and spermatozoa has been investigated by means of lectin cytochemistry. This has been combined with several chemical and enzymatic procedures to obtain more information.

The acrosome was a cell structure rich in carbohydrates. The spermatids showed O-linked oligosaccharides containing GalNAc residues positive to DBA and SBA, although with HPA, N-linked oligosaccharides with GalNAc residues were found. The spermatozoa showed a similar composition with respect to GalNAc, except that N-linked oligosaccharides with DBA-positive GalNAc residues were shown. GalNAc presence has been shown in the acrosome by SBA-lectin histochemistry in several mammals [7–9,11,12,33–40], and in insects [41]. However, in another report SBA did not bind to the acrosomal region of the rat [42]. DBA has also detected GalNAc in the acrosome in other mammals [9,43]; showing in some cases, a high variability during spermiogenesis. This lectin is reactive at the early stages of acrosome development, losing its affinity in the middle stages of development and returning reactive when the sperm is developed.

Fuc residues were also localized in the acrosome with AAA; thus, Fuc residues were part of N- and O-linked oligosaccharides. Additionally, UEA-I-positive Fuc residues included in N-linked oligosaccharides were found in the acrosome of round spermatids. Oligosaccharides with Fuc residues have also been shown by UEA-I in the immature acrosome of the rat [7,38] and bull [8], and the presence of Fuc by means of *Pisum sativum* agglutinin (PSA) has been reported in the inner acrosomal membrane of the goat [44]. Interestingly, in some mammalian species, like the nutria, mouse and gerbil, acrosomal UEA-I-labeling is highly variable during spermiogenesis [9]. No affinity of UEA-I lectin for the acrosome of guinea pig [9] and the musk shrew [11] has been reported. However, the guinea pig proacrosin, the zymogen precursor of acrosin, a sperm acrosomic protease believed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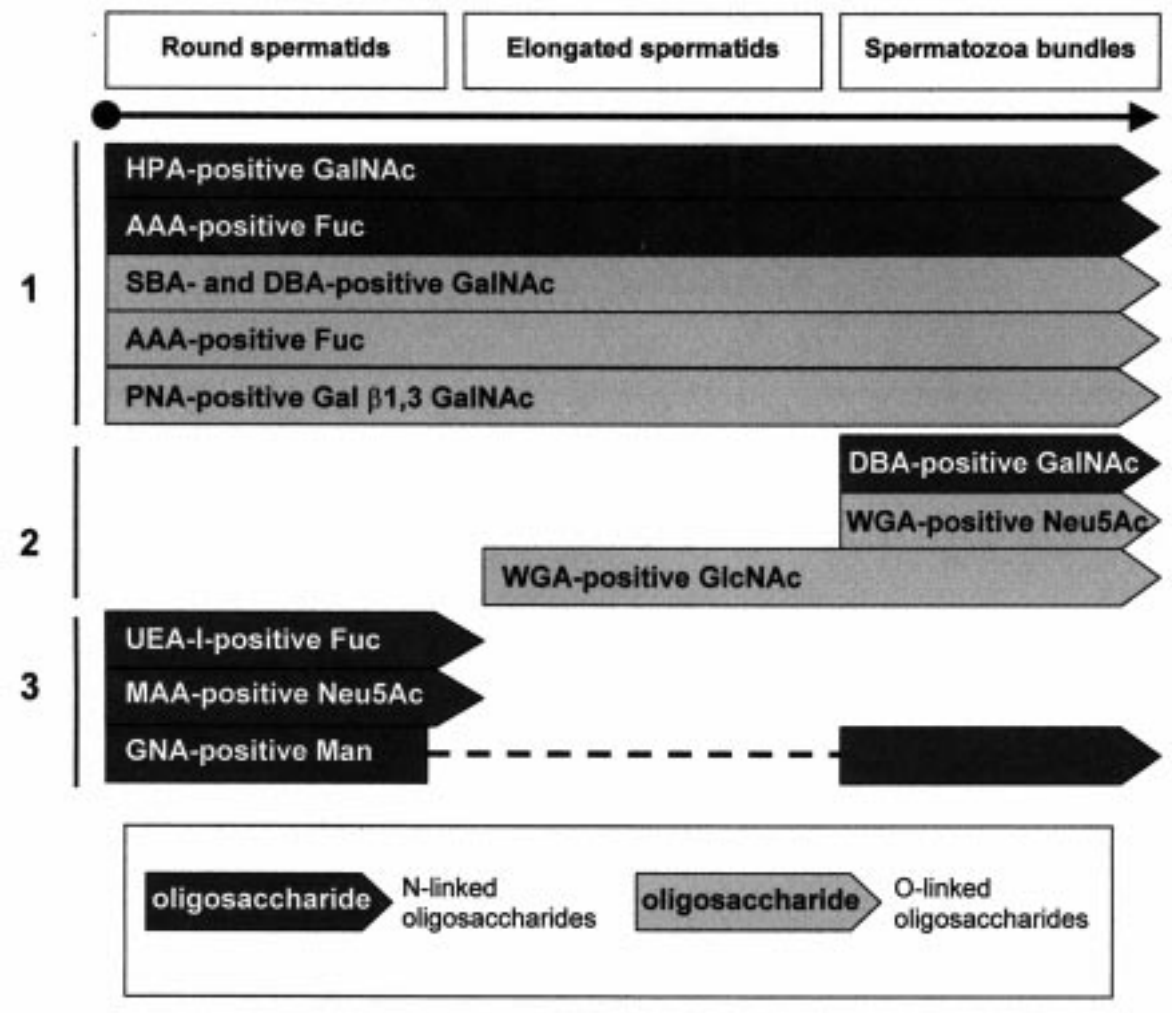


**Figure 7.** Schematic representation showing the terminal carbohydrates localized by lectin histochemistry into the acrosome during the spermiogenesis of *P. waltl*. Spermiogenic process is represented from the earliest steps (round spermatids, left) to the latest ones (spermatozoa bundles, right). Carbohydrates are classified into three groups: some of them are localized into the acrosome during all the spermiogenic stages (1), other carbohydrates added to the acrosome of the spermatozoa (2), and those present in the round spermatids that are not labeled at some of the most matures stages (3). When the localization of a terminal carbohydrate on O- or N-linked oligosaccharides cannot be clearly inferred, it has been omitted.

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**Figure 1.** SBA histochemistry (1a) and SBA histochemistry after $\beta$-elimination pre-treatment (1b) of testicular lobules containing elongated spermatids that are all packed together, with their ends directed towards the nucleus of the follicular cells (asterisks). In 1a, the acrosomes (arrows) are stained at the point of the elongating head spermatids. No labeled structure can be seen at the sharp end of the spermatids in 1b. Tails are positive in both 1a and 1b (arrow heads). Counterstained with hematoxylin. 650×.

**Figure 2.** HPA lectin histochemistry (2a) and HPA lectin histochemistry after PNGase-F pre-treatment (2b). In 2a, there is a cyst with his follicular cell (asterisk) and early elongated spermatids (2a). An intense labeling of the acrosomes can be seen (arrows), while the tails are not stained (arrow head). In 2b, the acrosomes (arrows) and the tails of spermatids (arrow heads) are stained. Counterstained with hematoxylin. 650×.

**Figure 3.** Round spermatids and their accompanying follicular cells (asterisks) in a lobule after lectin histochemical treatment with AAA. In addition to a cytoplasmic staining, a strong labeling of the proacrosomal granules (arrows) can be seen. Counterstained with hematoxylin. 650×.

**Figure 4.** PNA-lectin labeling (4a) and $\beta$-elimination pre-treatment and PNA-lectin labeling (4b) of a lobule containing round spermatids. In 4a, labeled acrosomes can be seen in the cytoplasm (arrows). Very weak or no staining in the acrosomes can be seen in 4b. Counterstained with hematoxylin. 650×.

**Figure 5.** A testicular lobule with round spermatids showing SNA-lectin-labeling in the tails (arrow heads). Counterstained with hematoxylin. 250×.

**Figure 6.** GNA-lectin labeling of testicular lobules with round (6a) and elongated spermatids (6b) and their follicular cells (asterisks). The acrosomes are moderately stained only in the round spermatids (arrows). Counterstained with hematoxylin. 650×.

to play an essential role in fertilization, is a glycoprotein that contains Fuc residues [45]. Moreover, the presence of Fuc in the guinea pig acrosome has been demonstrated by studying the distribution of $^{3}$H-fucose in autoradiographs [46].

Acrosomal Gal residues were only demonstrated with PNA, and they were part of O-linked oligosaccharides. In the lizard *Podarcis campestris* testis, the acrosome always has N-linked oligosaccharides with Gal terminal residues, while in O-linked oligosaccharides Gal is present only during the reproductive period [15]. Gal is present in the acrosome of the spermatids of other animals, as demonstrated by PNA and *Ricinus communis* (castor bean) agglutinin-I (RCA-I) in several mammalian species [7–9,11,12,23,33,36–38,40,47], or only by PNA staining in insect spermatogenetic cells [41].

For the three specific sialic acid binding lectins, this carbohydrate was absent in the acrosome of *Pleurodeles waltl* spermatids and spermatozoa. Only a small amount of Neu5Ac$\alpha$2,3Gal$\beta$1,4GlcNAc in N-linked oligosaccharides was unmasked after the $\beta$-elimination procedure. However, with WGA some sialic acid residues were localized in the acrosome of spermatozoa, as was manifested by the absence of reactivity after acid hydrolysis treatment (Table 2). Sialic acid is also present in the acrosome of lizard [15].

Some GlcNAc residues located in O-linked oligosaccharides were found in the acrosome of elongated spermatids. GlcNAc, like Fuc, has been shown to be another carbohydrate residue in the guinea pig proacrosin [45].

The acrosome of round spermatids and spermatozoa were positive for ConA, a pattern that depended upon the pre-treatment used. The staining disappeared after the glucose-oxidase procedure, the labeling can therefore be attributed to Glc residues. Only the acrosome of early spermatids and spermatozoa, but not that of the late spermatids, in the nutria, gerbil, mouse and guinea pig were positive for ConA [9]. Moreover, triantennary high mannose oligosaccharides are present in acrosome of rat sperm [22]. Interestingly, GNA-positive labeling was observed in round spermatid and sperm acrosome, but not in elongated spermatids.

In agreement with our observations, in another amphibian, *Bufo calamita*, a variability between spermatid and sperm acrosomal glycan composition was reported by means of lectin histochemistry [14]. Variable patterns of lectin staining in acrosome during spermiogenesis has also been reported in several mammalian species [7,9,11,12,23,33,40,47]. The identification of new carbohydrates in the acrosome during sperm maturation suggest the sequential incorporation of new components, indicating the existence of a pre-determined pattern of storage. On the other hand, the identification of some carbohydrates only in the acrosome of round spermatids suggest a progressive compression to facilitate the storage of the new components. As a consequence of this compressing process some carbohydrates are not accessible to lectins in the more mature spermatids and spermatozoa.

The tails of the spermatids and spermatozoa contained oligosaccharides composed of GalNAc, Fuc, Gal$\beta$1,3GalNAc (in O-linked oligosaccharides), Gal$\beta$1,4GlcNAc, Neu5Ac$\alpha$2,3Gal$\beta$1,4GlcNAc, Neu5Ac$\alpha$2,6Gal/GalNAc and GlcNAc. Man residues were not clearly demonstrated; GNA was negative and ConA was slightly positive, increasing the staining after glucose-oxidase treatment.

This work provides the first detailed characterization of the N- and O-linked oligosaccharides components during spermiogenesis in the testis of a urodele amphibian. The most interesting results are summarized in Figure 7, and concern the terminal carbohydrates detected on oligosaccharide chains of the acrosome, which include terminal GalNAc on N-linked glycans. Although the latter finding is unusual, it has been previously suggested [48]. In this vesicle, some terminal carbohydrates are observed in all stages of spermiogenesis, while others are seen only in the earliest or in the latest stages. The label variability during spermiogenesis found in this work indicates that acrosomal glycoconjugates could be modified during acrosome development in two ways: 1) addition of new carbohydrates, and 2) compression of acrosomal components that results in masking of some sugar residues.

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