**Introduction**

One of the most frequent ocular pathologies worldwide is corneal inflammation, or keratitis, which may be associated with infectious processes (Mellado et al. 2013). While noninfectious keratitis is generated by minor trauma, minor injury, or the use of contact lenses, infectious keratitis is the most prevalent cause of corneal blindness worldwide, and it is a consequence of infections by bacteria, viruses, fungi, or amoeba (Darren et al. 2021). Fungal keratitis, sometimes called keratomycosis, is caused by a broad spectrum of microorganisms, which differ depending on the geographical area involved. For this reason, yeasts are recognized as the most frequent causal agents in temperate climates, especially those of the *Candida* genus, with *Candida albicans* being the most common. However, in tropical climates, the main infectious agent of fungal keratitis are filamentous fungi (Mellado et al. 2013; Thomas and Kalamurthy 2013).

The identification of the epithelial receptors which enable adherence and colonization is critical concerning fungal keratitis, as it is in any other infectious process. These receptors, which are carbohydrates, proteins, or mixed molecules, are also essential for the microbial invasion that follows and the initiation of the immune response in the host (Wilson et al. 2002). Previous studies have shown that glycosaminoglycans (GAGs) serve as receptors for the adhesion of fungi.
from corneal cell surfaces are involved in the initial adherence of pathogenic bacteria, but that the process differs depending on their Gram-type (García et al. 2016b). GAGs are polysaccharide chains that usually form part of a proteoglycan; their composition differs depending on the disaccharide of which they are composed, either N-acetylgulosamine (GlcNAc) or N-acetylgalactosamine (GalNAc), attached to glucuronic acid (GlcA) or galactose (Iozzo and Schafer 2015). Based on the structure of their disaccharides, GAGs are divided into four types: hyaluronic acid, keratan sulfate, heparan sulfate (HS), and chondroitin sulfate (CS), the last two being the most important in terms of the cell surface and the pericellular matrix. Both contain a GlcA residue, GlcNAc in the case of HS, and a GalNAc residue in that of CS. During their polymerization, the HS and CS chains undergo modifications, which provide molecules with a complex structure dependent on the tissue and the physiological or pathological state of the cells involved (Kjellén and Lindahl 1991; Iozzo and Schafer 2015). HS chain residues can suffer N-deacetylation/N-sulfation of the GlcNAc, epimerization and/or O-sulfation of C2 in GlcA, and sulfations of C3 and C6 in GlcNAc, while modifications to the CS chains are simpler: GlcA can be epimerized and sulfated at C2, while GalNAc can be sulfated at C4 and C6. As a result, HS chains have high sulfate, heparan sulfate (HS), and chondroitin sulfate (CS), all from Sigma-Aldrich (St. Louis, MO, USA); the yeast extract culture medium was from Conda (Spain); Dulbecco’s Modified Eagle’s Minimal essential medium (DMEM), fetal bovine serum (FBS), insulin, epidermal growth factor (EGF), penicillin G/streptomycin, and phosphate-buffered saline (PBS) were all from Gibco (Life Technologies, CA, USA). Fungal strains, cell lines, and culture conditions. The fungal species used in this study were C. albicans, Candida glabrata and Candida parapsilosis, all clinical isolates obtained from the Central University Hospital of Asturias and identified at the species level by MALDI-TOF MS spectrometry (Bruker Daltonics, Bremen, Germany). All were grown in Saboureaud medium with chloramphenicol at a concentration of 50 µg/l at 28°C for 24 h, except C. albicans in its fermented form, which was grown in medium containing 0.1% glucose, 1% glycerine and 0.1% yeast extract at pH 7.5 at 37°C in a 5% (v/v) CO₂ atmosphere for 48 h. HCE-2 [50.B1] ATCC CRL-11135 corneal cell line was grown in DMEM supplemented with 10% FBS, 1.5 units/ml of insulin, 10 ng/ml of EGF and penicillin G/streptomycin (5,000 IU/ml, 5,000 µg/ml). Cultures were incubated at 37°C in a 5% (v/v) CO₂ atmosphere.

Fluorescence labeling. Overnight fungal cultures were washed four times with PBS buffer, resuspended in a 0.1 mg/ml FITC solution to an A₆₀₀ of 0.5, and incubated in the dark at 37°C under agitation for 1 h. After that, the excess of FITC was removed by four washes with PBS. Finally, the pellet was resuspended in DMEM.

Inhibition of glycosaminoglycans biosynthesis. Cell cultures were grown in 24-well plates to 70% confluence and incubated overnight at 37°C in DMEM containing either rhodamine B at 50 µg/ml or genistein at 30 µM. Wells were then washed twice with PBS, and the cell integrity was verified under an optical microscope before being used in the adhesion assays.

Enzymatic digestion of cell surface glycosaminoglycans. The hydrolysis of the GAGs on the surface of the corneal cells was carried out by incubating for 3 h at 37°C in a 5% CO₂ atmosphere with a 500 mU/ml (final concentration) mix of heparinase I and III for Experimental Materials and Methods Materials. The materials were purchased from the following manufacturers: Saboureaud culture medium from Difco (BD, MD, USA); the antibiotic chloramphenicol, fluorescein isothiocyanate (FITC), rhodamine B, genistein, heparinase I and III, chondroitinase ABC, HS, chondroitin sulfate A (CS A), chondroitin sulfate B (CS B), and chondroitin sulfate C (CS C), all from Sigma-Aldrich (St. Louis, MO, USA); the yeast extract culture medium was from Conda (Spain); Dulbecco’s Modified Eagle’s Minimal essential medium (DMEM), fetal bovine serum (FBS), insulin, epidermal growth factor (EGF), penicillin G/streptomycin, and phosphate-buffered saline (PBS) were all from Gibco (Life Technologies, CA, USA).
HS and 250 mU/ml (final concentration) of chondroitinase ABC for CS. Digestion of both GAG species was achieved through successive incubations of the cell cultures with the two enzymatic mixes, washing between each with PBS. The reactions were stopped through two washes with PBS. Cell integrity was verified by light microscopy before carrying out the adhesion assays.

**Adhesion assays.** Adhesion of the different fungi to HCE-2 monolayers was performed in 24-well plates to 70–90% confluence. The medium was removed, and the cells were washed twice with PBS and then blocked with 10% FBS in DMEM for 1 h at 37°C in a 5% CO₂ atmosphere. After that, the cells were washed with PBS, and 200 µl of FITC-labeled yeast in 300 µl of DMEM were added. The mixture was incubated for 90 min at 37°C in a 5% CO₂ atmosphere. To remove the unbound fungi, wells were rinsed four times with 500 µl of PBS. At the end of the experiment, HCE-2 cells were disaggregated with 1% SDS, and the fluorescence of the yeast attached to them was quantified in a Perkin Elmer LS55 fluorometer (Perkin Elmer, MA, USA), at 488 nm for excitation and 560 nm for emission. Values obtained for the different experiments were normalized using the adhesion value without any treatment as a reference, which was given a value of 100%.

For adherence inhibition assays, FITC-labeled yeast was co-incubated with HS, CS A, CS B, CS C, or a mixture of the four, at concentrations ranging from 0.05 to 2.5 µg/ml for 30 min. After this, adhesion assays were performed as indicated above.

**Statistical analysis.** All experiments were performed at least three times and with a minimum of three replications in each case. The data were analyzed with the Statistica program for Windows (Statsoft Inc., OK, USA). The mean values of two samples were compared using the Mann-Whitney U test, with p < 0.05 being considered as significant.

**Results**

**GAGs are involved in the adherence of different Candida species to corneal epithelial cells.** To analyze whether GAGs play a role during the binding of Candida spp. to corneal epithelial cells, the synthesis of cell surface GAGs was inhibited using either rhodamine B or genistein. The results showed a decrease in fungal binding after treatment with either inhibitor, suggesting that GAGs are involved in adherence to the corneal epithelium. The effects varied depending on the fungus and the inhibitor. Adherence of *C. albicans* was strongly reduced after treatment with both rhodamine B and genistein, decreasing by 80% and 50%, respectively (Fig. 1a). However, in the *C. glabrata* and *C. parapsilosis* experiments, rhodamine B generated a statistically significant decrease of 30% in the binding of both fungi, while genistein produced a reduction of 70% in *C. glabrata* and 50% in *C. parapsilosis* (Fig. 1a).

To investigate the role of GAGs on fungal adherence more deeply, the corneal cell surface GAGs were degraded using the bacterial lyases heparinase I and III and chondroitinase ABC, which degrade HS and CS, respectively. After treatment with these lyases, the adherence of all the species of *Candida* analyzed was reduced to a statistically significant degree, reinforcing the notion that both HS and CS are involved in their binding to the corneal epithelial cell surface.

Treatment with heparinase I and III similarly reduced the adherence of *C. albicans* and *C. glabrata*, by around 60%, but chondroitinase ABC produced a decrease of 45% (Fig. 1b). It appears to indicate that HS preferably mediates the binding. The degradation of both GAG species by the combined use of the lyases reduced the adherence of *C. albicans* by 70%, suggesting the existence of a cooperative effect. However, this effect was not observed in *C. glabrata* (Fig. 1b). On the other hand, degradation of HS and CS or mixture of the two molecular species produced a similar effect on the adherence of *C. parapsilosis*, reducing the binding of the fungus to corneal cells by approximately 40% (Fig. 1b).

**Role of GAGs on the adherence of C. albicans in its two morphological forms: yeast and hyphae.** To investigate whether GAGs are also involved in the binding of filamented *C. albicans*, the same tests as in the section above were carried out, and the results compared with those of their yeast morphology (Fig. 2). Unlike the inhibitory effect that rhodamine B and genistein...
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Effect of reducing cell GAGs on the adhesion of *C. albicans* yeast cells and hyphal cells to corneal epithelial cells. a) Inhibition of fungal adhesion to HCE-2 cells treated with rhodamine B (black bars) and genistein (gray bars). b) Inhibition of fungal adhesion to HCE-2 cells treated with heparinases I and III (black bars), chondroitinase ABC (dark gray bars), or a mix of heparinases I and III + chondroitinase ABC (gray bars). Data were normalized using the adhesion values of fungi adhesion to non-treated cells, given the arbitrary value of 100. Error bars represent standard deviations. Statistically significant differences are denoted by *, which indicates $p < 0.05$.

Differential involvement of specific GAGs on the adherence of *Candida* species to corneal epithelial cells. To analyze the role of different species of GAGs on the adherence of *C. albicans*, *C. glabrata* and *C. parapsilosis* to the corneal epithelium, commercial GAGs were used (HS, CS A, CS B, CS C, and a mixture of all of them) as competing molecules. A decrease in yeast binding to corneal cells was observed in the presence of each of the GAGs, the effect being dependent on the yeast involved.

In the case of *C. albicans*, the CS molecules were most effective at lower concentrations than HS, and CS A was the most efficient, followed by CS B and CS C (Fig. 3a). In contrast, at higher concentrations, HS was the most effective interfering molecule, followed by CS B (Fig. 3a), although none of the interfering molecules was able to reduce the adherence of *C. albicans* by more than 20%. However, the mixture of GAGs showed a higher competitive capacity, which was dependent on the concentration, inhibiting binding by about 40% at a concentration of 2.5 μg/ml (Fig. 3a).

Experiments with *C. glabrata* showed that CS B and CS A were the least efficient molecules, reducing yeast binding by approximately 15% at the highest concentrations, while HS and CS C achieved inhibitions of 30% (Fig. 3b). In this case, the combined use of a mixture of GAGs did not increase the inhibitory effect concerning that caused by each species individually (Fig. 3b).

GAGs also interfered in the adhesion of *C. parapsilosis* to corneal cells. HS and CS B were less efficient as interfering molecules, with only a 15% reduction in yeast binding at the highest concentration tested (Fig. 3c). CS C was the strongest inhibitor, reaching values close to 30% (Fig. 3c), while CS A had a slightly lesser effect, although at high concentrations, it interfered with the binding of *C. parapsilosis* to the same extent as CS C (Fig. 3c). When the mix of GAGs was used, the observed effect was stronger, reaching an inhibition of nearly 60% (Fig. 3c).

The implication of specific N- and O-sulfations on the adherence of different *Candida* spp. to corneal epithelial cells...
epithelial cells. To analyze how sulfations of the HS chains might influence the adherence process, we carried out experiments using HCE-2 cell lines where the specific sulfotransferases involved in these sulfations were silenced using interference RNA (García et al. 2016b). Only the adhesion of C. albicans and C. glabrata to corneal cells was reduced in these experiments (Fig. 4). A decrease in N-sulfation produced a statistically significant reduction in the binding of C. albicans and C. glabrata, of around 20% and 30%, respectively (Fig. 4). A reduction in 2-O-sulfation led to a statistically significant decrease of 20% in the adhesion of C. glabrata, while a statistically significant increase in the binding of C. parapsilosis was found (Fig. 4). On the other hand, a reduction in 6-O-sulfation only produced a statistically significant reduction, of around 40%, in the binding of C. albicans (Fig. 4).

Discussion

Fungal keratitis is one of the most frequent pathologies seen in ophthalmology. The corneal epithelium is the first barrier to the entrance of microorganisms as it is the first binding point in infectious processes. Candida spp. is the most common agent that produces eye infections, ocular trauma being the most frequent predisposing factor (Lakhundi et al. 2017). GAGs are involved in the initiation of bacterial pathogenesis, acting as receptors in, among others, lung, corneal, and epithelial tissue (García et al. 2016a; Martin et al. 2019; Rajas et al. 2017). In addition, the disaccharide composition of GAG chains and their degree of sulfation depend on the type and physiological state of the cell (García et al. 2016a). This is of great importance in analyzing GAGs as receptors in fungal keratitis.

The role of GAGs in the adhesion of C. albicans, C. glabrata, and C. parapsilosis to corneal epithelial cells was analyzed through the inhibition of their synthesis using either rhodamine B or genistein. The effects differed depending on the fungus involved. Both inhibitors significantly reduced the binding of the yeasts, although rhodamine B produced greater effects in C. albicans, as it has also been observed in other cell types such as keratinocytes of the epidermis (Ordiales et al. 2021). Genistein was more effective than rhodamine B in inhibiting the binding of C. glabrata and C. parapsilosis. The differences observed between the inhibitors may be the result of intrinsic characteristics of each yeast species, such as the expression of different adhesins, but could equally be due to the different mechanisms of action of the two inhibitors (Kaji et al. 1991; Nikitovic et al. 2003; Piotrowska et al. 2006; Roberts et al. 2006; Silva et al. 2012; Ordiales et al. 2021). Interestingly, the results were very different in the experiments using C. albicans hyphae, with an increase in its adherence after treatment with the inhibitors. This effect has also been observed in fibroblasts of the dermis, where C. albicans hyphae binding increased after treatment with both rhodamine B and genistein (Ordiales et al. 2021).

The enzymatic elimination of GAGs from the cell surface through bacterial lyases caused a significant
decrease in fungal adherence in all cases except, once again, with the filamented form of C. albicans. Moreover, the adherence of C. albicans and C. glabrata was more reduced when heparinas were used, indicating that HS may well be the main GAG species involved in the early stages of infection. On the contrary, the effect of the enzymatic degradation of the different GAGs reduced the adhesion of C. parapsilosis to a very similar degree, regardless of the lyase involved. It should be noted that the common treatment of heparinas and chondroitinases only had a combined effect in the case of C. albicans. What is more, the complexity of the GAGs, the possibility of them forming ternary complexes with other receptors, and the possibility that digestion was incomplete or that compensatory mechanisms were activated could explain these differences. Also, when C. albicans is in filament form, its interaction with corneal cells is no longer dependent on GAGs since adherence increases when GAGs are eliminated from the cell surface. This fact may point to the existence of other receptors with a high affinity for C. albicans hyphal adhesins, which would be more exposed to binding after the elimination of the GAGs.

Moreover, changes in morphology could also involve the expression of other adhesins necessary for colonization and perhaps display a high affinity for different receptors. The data as a whole seems to suggest that, at least in part, pathogenic yeasts use GAGs as receptors and that this phenomenon depends on the molecular species involved. As was mentioned earlier, it has been previously described that bacteria use GAGs to bind to the corneal epithelium, showing different patterns depending on their Gram nature (García et al. 2016b). It also has been shown that C. albicans has a preference for CS as a receptor in the keratinocytes of the epidermis. It contrasts with the findings of the present work, thus reinforcing the importance of the final structure of the GAG chains involved in the adhesion of microorganisms (Ordiales et al. 2021).

The involvement of different species of GAGs in fungal adhesion was evaluated through competition with commercial molecules. Inhibition of binding was obtained to a variable degree depending on the interfering molecule and the yeast used. HS and CS B showed the most significant inhibition in C. albicans, followed by CS A and CS C. However, the effect was opposite in C. parapsilosis, with CS C being the most interfering molecule. CS B, unlike other species of CS, presents iduronic acid residues in variable proportions along its chain as a result of GlcA epimerization, which provides the molecule with greater flexibility (Thelin et al. 2013). The epimerization at C5 of GlcA also occurs in HS chain. Additionally, CS A and CS C are sulfated at C4 and C6 of GalNAc respectively, although CS B and HS are usually more sulfated. These characteristics may explain the results, epimerization being of paramount importance in the adhesion process of C. albicans, as is the sulfation of GalNAc in C. parapsilosis. The mix of GAGs had a combined effect, increasing the inhibition of both these yeasts, and although greater in C. parapsilosis, total inhibition was not achieved in either case, suggesting the existence of other receptors. One reason for the differences could be that the affinity of the yeasts for commercial GAGs may vary and could even be lower than when they interact with the GAGs of the corneal cells.

Furthermore, GAGs can frequently form complexes with other ligands or receptors. The epimerization of GlcA and the sulfation of GalNAc do not seem to be crucial for binding in C. glabrata. HS and CS C present a similar degree of binding inhibition, with CS A and CS B being the least interfering molecules. Furthermore, the mix of GAGs did not result in greater inhibition. Taken together, all the data point to the importance of GAGs as cell receptors in fungal adherence.

The complex and dynamic structure of GAG chains depends on the cell's type, location, and physiological state, allowing them to participate in a multitude of pathological processes, including infections. The affinity of bacteria for different species of GAGs during their pathogenesis has been widely described (Zaretzky et al. 1995; Leong et al. 1998; Tonnaer et al. 2006; Kobayashi et al. 2011; Ordiales et al. 2021).

To investigate the influence of sulfation at specific positions on adherence, we used corneal cell lines previously developed in our laboratory where the genes specifically involved in introducing sulfate groups at specific positions of the disaccharide were silenced (García et al. 2016b). The design of these cell lines took into account the inhibition of all the genes encoding isoforms responsible for the same type of sulfation ensured that the transcription of the rest of the genes involved in biosynthesis was not affected since multiple isoforms are responsible for some of these sulfations, along with the possible existence of space-time regulated enzyme complexes, both at the transcriptional and translational levels (Víctor et al. 2009). The reduction of sulfation at different positions affected the attachment of the fungi differently, and no common patterns of inhibition of adherence were observed. The binding of C. albicans was very sensitive to 6-O sulfation of GlcNAc, although N-sulfation in this residue only affected the binding of C. albicans and C. glabrata. In addition, the 2-O sulfation of uronic acid residue only inhibited the binding of C. glabrata, the opposite of what happened in C. parapsilosis experiments. Despite the importance of GAG chain sulfation patterns in the binding of Candida spp. to ligands, few studies related to their influence on binding to microorganisms have been published. However, the importance of N- and 6-O sulfation of GlcNAc in corneal infections has been demonstrated, along with
the fact that 2-O sulfation of uronic acid residues has less importance (García et al. 2016b).

In conclusion, our data seems to indicate that GAGs act, at least in part, as receptors for the binding of C. albicans, C. glabrata, and C. parapsilosis to corneal epithelial cells although there are differences between fungal species. In the case of C. albicans, its morphological form is responsible for radical differences in terms of its dependence on GAGs as receptors, probably due to differences in the composition of the cell wall in the different forms (Naglik et al. 2011), which would lead to changes in the expression of adhesins. This fact might cause alterations in its affinity to GAGs and modifications in its binding to other receptors. These results approximate what can happen in vivo, although other factors about the cellular environment and the cell itself should be taken into account for future research. The results of this work open the door to new strategies that can inhibit the adhesion and invasion of pathogenic fungi to the cornea, thus reducing the possibility of developing keratitis.

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**Conflict of interest**

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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