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OCULOPLASTICS AND ORBIT

Study of conjunctival flora in anophthalmic patients: influence on the comfort of the socket

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Abstract
Purpose To investigate the relationship between conjunctival flora and comfort of the socket in anophthalmic patients.
Methods A cross-sectional clinical study including 60 patients with unilateral anophthalmia who wear a prosthetic eye. From each patient three microbiological samples were taken from the lower conjunctival sac (healthy eye, pre-prosthesis, and retro-prosthesis space of socket). The 180 samples obtained were cultured. Samples from a randomized subgroup of 29 patients were measured by spectrophotometry at 540 nm after 48 h of growth, to determine their microbial density (MD). The grade of comfort of the socket (GCS) of each patient was established by a questionnaire. Epidemiological and clinical data of the anophthalmic socket and artificial eye care of each patient were also collected.
Results MD decreased in healthy eyes (0.213 ± 0.201, $P = 0.004$) compared with the pre-prosthesis (0.402 ± 0.323) and retro-prosthesis (0.438 ± 0.268) samples. Pre-prosthesis MD correlated with retro-prosthesis MD ($R = 0.401$, $P = 0.031$) and healthy eye MD ($R = 0.482$, $P = 0.008$), and it was also related to poor GCS ($P = 0.017$). Aerobic Gram-negative bacteria in retro-prosthesis samples of patients with poor GCS was higher than in patients with good or fair GCS ($P = 0.008$). In the same samples, coagulase-negative staphylococci proportion (excluding *S. epidermidis*) increased in patients with good GCS ($P = 0.030$).
Conclusions Socket microflora is related to GCS. Increased pathogenic flora, especially Gram-negative bacteria, and high MD are related to discomfort, while coagulase-negative staphylococci (other than *S. epidermidis*) are associated with comfort.

Keywords Socket · Ocular prosthesis · Discomfort · Orbital implant · Microflora · Conjunctival dysbiosis · Microbial density · Spectrophotometry · Turbidimetry

Introduction

Ocular prostheses improve the significant esthetic alteration that results from the loss of an eye. However, ocular prosthesis wearers experience very variable degrees of comfort, and often they present symptoms of chronic discharge and irritation [1].

Different factors have been suggested as the root of these problems: poor fitting of the prosthesis [2], the removal regime [3], and Meibomian gland dysfunction [4]. Some attempts have also been made to link the conjunctival flora present in the socket with the complaints of anophthalmic patients [5–9]. Since dysbiosis in the other most frequently studied epithelia—for example, intestinal [10–12], cutaneous [13, 14], or vaginal [15] epithelia—are related to diseases, it seems reasonable that conjunctival dysbiosis may be associated with changes in the comfort of the ocular prosthesis [16].

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The bacterial flora of the conjunctiva has been widely examined in healthy subjects [17–21]. This normal flora is formed mainly by *Staphylococcus epidermidis* and coryneform bacteria. Several studies have analyzed the conjunctival flora in anophthalmic sockets [5–9, 22–25] and generally have shown higher rates of pathogens in sockets compared to the normal conjunctival flora. Reports comparing the flora of the socket with the fellow eye tend to exhibit a greater presence of pathogens in the socket relative to the healthy eye [5, 8, 16, 22, 23]. Moreover, some of these studies suggest that the flora of the anophthalmic socket affects the healthy eye flora, and they therefore recommend maximizing the anti-infective prophylaxis of the fellow eye during intraocular surgery [8, 22, 23]. Nevertheless, the correlation between the 2 floras has not been pinpointed. Regarding the relationship between comfort and the flora of the socket, studies have shown conflicting outcomes [5–9]. In addition, no study has evaluated the orbital implant influence on the conjunctival flora of the socket, nor has it been assessed whether there are differences in conjunctival flora contained between the eyelid and the artificial eye (pre-prosthesis flora) and between the artificial eye and the socket (retro-prosthesis flora).

This study aims to answer these questions by performing an identification of the conjunctival flora of anophthalmic patients in both the socket and the healthy eye, and by analyzing potential differences and relationships between the floras of the different conjunctival spaces. We subsequently assessed whether some particular groups of microorganisms are related to socket comfort. Other factors such as orbital implants, frequency of prosthetic eye removal, or routine use of topical antibiotics in the socket were also analyzed in relation to socket comfort.

Methods

This study was approved by the institutional review board of our hospital. The techniques used to collect the data conformed to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all study patients before participation.

Patient selection

A total of 60 anophthalmic patients were included in this study. The inclusion criteria were subjects with a history of unilateral anophthalmia, daily wearing of an artificial eye, and stable symptomatology in the socket for a minimum of 1 month. Patients that regularly applied antibiotic eyedrops in their sockets without prescription were also included if they had used the medication during the last month.

Exclusion criteria comprised any type of acute conjunctivitis, conjunctival cyst, orbital implant exposure, or use of a scratched or poorly fitting prosthesis. Patients with phthisis bulbi and cosmetic scleral shells were also excluded, since they are not anophthalmic.

Clinical examination

All patients were examined by the same ophthalmologist. Demographic and health information were collected for each patient, including age, gender, anophthalmic side, health status, and use of systemic or topical medications. Hospital records supplied the date and cause of eye loss; the type of procedure (evisceration vs. enucleation); and the presence of an orbital implant and its shape, material, and size. The grade of comfort of the socket (GCS) of each patient was assessed by a questionnaire (see GCS Assessment section). In addition, patients provided information on longevity of the ocular prosthesis, frequency of removal, and socket watering. After collecting the samples, canalicular irrigation was performed in patients with epiphora to determine if there was obstruction of the lacrimal pathway.

Finally, the socket was examined for signs of inflammation when microbiological samples had already been collected and the ocular prosthesis had been removed. Mucus in the socket was categorized as a normal (sparse and transparent) or abnormal (profuse and colored). The conjunctiva of socket was also classified as healthy or hyperemic. Furthermore, a routine eye examination using slit-lamp biomicroscopy was performed on the healthy eye.

Grade of comfort of the socket assessment

All the participants self-responded to a questionnaire to evaluate GCS (Supplemental Material 1). This document consisted of 4 questions assessing the frequency in the anophthalmic socket during the last month of (1) abundant dry rheum; (2) tearing; (3) mucus discharge; or (4) symptoms as itching, burning, or foreign body sensation. Similar to the OSDI questionnaire, each question was graded on a scale from 0 ("none of the time") to 4 ("all of the time") [26]. The scale ranged from 0 to 16 points, where lower scores indicated better comfort in the socket. The second question was eliminated in patients with epiphora who showed lacrimal pathway obstruction. In these cases, a proportional score for 4 questions was calculated based on the points scored in the other 3 questions. This calculation is necessary, as otherwise the GCS would be artificially moved in patients with lacrimal pathway obstruction. A sum of scores for all responses was used to allocate GCS into 3 categories: good (0 to 5 points), fair (6 to 10 points) or poor (11 to 16).

Conjunctival flora identification

Specimens were obtained by the same ophthalmologist using a uniform technique. To avoid damaging the microorganisms

of the conjunctival flora, topical anesthetic was not used. Three microbiological samples were taken of each patient from the lower conjunctival sac of (1) the healthy eye; (2) the anophthalmic socket before removing the ocular prosthesis (pre-prosthesis sample); and (3) the socket after carefully removing the artificial eye using a suction cup (retro-prosthesis sample).

Samples were performed using a sterile rayon swab (Copan Diagnostics Inc., Murrieta, CA, USA). The swab was previously moistened with sterile brain heart infusion (BHI) culture medium by introducing it for 2 s into a tube (Becton, Dickinson & Company, Franklin Lakes, NJ, USA) with only 4 mL of BHI. The swab was passed twice through the conjunctival sac, everting the lower eyelid and avoiding touching the lid margin. Afterwards, the swab was cut off and placed in the same tube used to moisten the swab, and the tube was closed. Samples were taken to the clinical microbiology laboratory of our hospital within an hour after collection.

The tubes with the samples were incubated at 37 °C in an aerobic atmosphere. They were examined 24 h after collection and then daily for 10 days before they were reported as negative-cultures. If growth was observed, the broth was subcultured to different media for microorganism isolation and identification. Culture media for fungi and aerobic, facultative anaerobic, and strict anaerobic bacteria were used. Microbial identification was based on growth in selective media, morphology of colonies, and the use of MicroScan (Siemens, Munich, Germany) or API (bioMérieux, Marcy l'Etoile, France) systems. Staphylococci were identified by employing panel 31 of the Microscan system, enterococci through panel 32, fermentative Gram-negative bacteria through panel 53, and non-fermentative Gram-negative bacteria through panel 54. The remaining microorganisms were identified through API galleries.

The isolated species were grouped to perform the statistical analysis. Groups that settled initially were staphylococci, streptococci, enterococci, *Micrococcus spp.*, coryneform bacteria [27], Gram-negative bacteria, and fungi. Since most of the bacteria isolated from the conjunctival flora were staphylococci, it was decided to divide this genus into 3 new groups: coagulase-positive staphylococci (CPS), coagulase-negative staphylococci (CNS) excluding *S. epidermidis*, and *S. epidermidis* which formed its own group. For analytical purposes, the presence or absence of microorganisms for each of these 9 groups was determined in each sample.

Furthermore, groups of microorganisms were classified as pathogens or saprophytes. CNS (*S. epidermidis* and other species), *Micrococcus spp.*, and coryneform bacteria were classified as saprophytic microorganisms. Other groups were considered pathogens. Each sample was classified as pathogenic flora if any microorganism belonging to the pathogenic groups had grown. Only tubes in which all isolated species fitted to saprophytic groups were allocated as saprophytic flora. When no microorganism was isolated, samples were considered negative-cultures.

Microbial density assessment

The microbial density (MD) or microorganisms per unit volume was quantified using turbidimetry [28]. An aliquot of BHI was taken from the sample after 48 h of culture, working under a laminar flow hood. It was then measured using spectrophotometry at 540 nm, employing a Thermo Helios Delta VIS (Thermo Electron Corporation, Waltham, MA, USA) spectrophotometer. To check if the manipulation performed during this measurement had somehow altered the cultured flora, only the samples of 29 randomly selected patients were evaluated. The 3 samples belonging to each selected patient were studied by spectrophotometry to determine their microbial density (87 tubes out of 180 obtained).

Statistical analysis

Results are presented as mean ± standard deviation for continuous variables distributed normally and as proportions for categorical variables. If a continuous variable was not distributed normally, a categorical transformation was performed, grouping the data into 2 groups, as in the cases of time since surgery, orbital implant size, longevity of the artificial eye, and removal regime. Comparisons of proportions between groups were made by Pearson's chi-square test with Yates's correction for continuity as appropriate. For hypothesis testing related to continuous outcomes, equal variance assumptions were checked using Levene's test. Where equal variance was not assumed, Welch's test was used to test associations between continuous variables and 2 categories. If equal variance was assumed, associations between continuous variables and 2 categories were tested with the Student *t* test, while for multiple categories they were performed through analysis of variance. In this case, many comparisons were completed, so the Bonferroni correction for multiple comparisons was used to adjust the *P* value. Linear regression with evaluation of the Pearson coefficient was performed to analyze associations between 2 continuous variables. A *P* value of less than 0.05 was considered to be statistically significant. Statistical analysis was carried out using SPSS for Windows version 22.0 (SPSS Inc., Chicago, IL, USA). AMOS 24.0 (SPSS Inc., Chicago, IL, USA) was used to perform confirmatory factor analysis. Charts were drawn with GraphPad Prism 5 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

Results

Study population

A total of 60 anophthalmic patients were examined. Men (60%) had a mean age of 61.4 ± 16.9 years, and women a mean age of 62.8 ± 15.8 years. The socket side was left in 36 patients. Regarding health status, 16 patients had no systemic medication. The most common diseases in the remaining 44 patients were arterial hypertension (68%), hypercholesterolemia (32%), diabetes mellitus (18%), and psychiatric depression (18%). Trauma (29/60) and endophthalmitis (16/60) were the most common causes of eye loss. Fifty-five patients had been operated on through ocular evisceration and only 5 through enucleation. Patients were classified according to two groups of time since surgery: a) operated on 20 years ago or less (38/60) and b) over 20 years ago (22/60). An orbital implant was present in the socket of 33 patients. All implants were round-shaped and made of Medpor® (91%) or silicone (9%). The implant diameters were 16 mm (3%), 18 mm (30%), 20 mm (24%), and 22 mm (42%).

All prostheses were acrylic plastic shells. The longevity of the 60 artificial eyes was classified into two groups: a) less than 1 year old (40%) and b) 1 year old or more (60%). Thirty-two patients admitted to removing their artificial eye from the socket at least once a week. These individuals were referred as "frequent manipulators". Regarding the routine use of topical products in the socket, 9 patients stated that they routinely employ self-prescribed antibiotic eyedrops, while the remaining patients either did not employ any product (34/60), or only lubricants (11/60) or saline (6/60). An abnormal mucus exudate was observed in 28 patients and conjunctival hyperemia of the socket in 17. Of the 14 patients that complained of epiphora, 8 had an obstruction of the lacrimal pathway.

Study of conjunctival flora

Conjunctival flora was obtained, cultured, and identified as described in the Methods section. The results of the microbiological isolation are shown in Table 1. The most commonly isolated species was *S. epidermidis*, with no differences between healthy eyes and socket samples ($P = 0.833$). Conversely, both streptococci ($P = 0.004$) and Gram-negative bacteria ($P = 0.036$) were significantly more frequent in sockets compared to healthy eyes. When microorganisms were grouped according to their pathogenicity, pathogenic flora samples prevailed in sockets ($P < 0.001$), whereas saprophytic flora ($P = 0.021$) and negative-cultures ($P = 0.004$) were more frequent in healthy eyes. There was no significant difference in conjunctival flora (by groups of microorganisms or type of flora) between samples taken from the pre-prosthesis and retro-prosthesis spaces. Likewise, the microbial composition of samples manipulated for measuring MD was similar to samples in which this parameter was not assessed.

When MD was analyzed, it was lower in samples of healthy eyes (0.213 ± 0.201, $P = 0.004$) compared to the pre-prosthesis (0.402 ± 0.323) and retro-prosthesis (0.438 ± 0.268) samples of the socket (Fig. 1). In addition, the MD of pre-prosthesis samples showed a positive statistical correlation with density of retro-prosthesis ($R = 0.401$, $P = 0.031$) and healthy eye samples ($R = 0.482$, $P = 0.008$). There was no correlation between the MD of retro-prosthesis samples and that of healthy eyes (Fig. 2).

Demographic and clinical characteristics of the patients were studied to assess their possible influences on the conjunctival flora. Patients' age and conjunctival inflammation in the socket were related to the flora of fellow eyes. Negative-cultures in healthy eyes corresponded to younger patients more than those with some microbial growth (51.9 ± 12.8 vs. 63.7 ± 16.4, $P = 0.044$). All the samples from healthy eyes of patients with abnormal mucus in their sockets had some microbial isolation (28/28 vs. 23/32, $P = 0.007$), and in those samples where MD was measured, it was higher than in patients without mucus in their sockets (0.295 ± 0.233 vs. 0.137 ± 0.131, $P = 0.037$). Moreover, patients with hyperemia in their sockets showed a greater proportion of pathogens in their healthy eyes (6/17 vs. 4/43, $P = 0.040$), without any particular group of microorganisms dominating.

Several clinical data (socket on left side, lack of systemic medication, surgery over 20 years ago, history of endophthalmitis, and absence of orbital implant) were related to increased streptococci in the anophthalmic socket, whether in the pre-prosthesis or retro-prosthesis samples (Table 2). When the flora was classified by pathogenicity, an increase of pathogenic microorganisms in pre-prosthesis samples was observed in patients with surgery over 20 years ago ($P = 0.016$) and without an orbital implant ($P = 0.005$). In retro-prosthesis samples, only those patients without an orbital implant showed an increase of pathogenic bacteria ($P = 0.035$). Since some of these clinical variables were associated with each other (such as orbital implant and time since surgery), a multi-factor analysis was performed to confirm their independent impacts on the type of flora. In the best fit model, the absence of an orbital implant was identified as the main factor related to increased pathogenic flora on the socket (Supplemental Material 2). Time since surgery was strongly associated to orbital implant, as patients who had been operated on recently were used to wearing an orbital implant unlike patients who had been operated on longer ago.

Other factors of the anophthalmic socket (epiphora, lacrimal pathway obstruction, and use of topical medications) were related to changes in this flora. All patients with epiphora showed growth of *S. epidermidis* in pre-prosthesis samples (14/14 vs. 29/46, $P = 0.019$) and also a higher MD in retro-prosthesis space (0.662 ± 0.246 vs. 0.380 ± 0.246, $P = 0.019$).

Table 1 Microflora isolated from sockets and fellow eyes of 60 patients

Conjunctival flora determinations	Healthy eyes	Anophthalmic socket		*P* Value‡
		Pre-prosthesis	Retro-prosthesis	
Microorganism groups*				
S. epidermidis	40	43	42	0.833
Others CNS	8	8	13	0.358
CPS	5	11	11	0.208
Coryneform bacteria	3	2	2	0.867
Micrococcus spp	1	1	0	0.442
Streptococci	2	14	13	0.004
Enterococci	1	2	1	0.786
Gram-negative bacteria	2	10	10	0.036
Fungi	0	1	1	0.442
Type of flora†				
Pathogenic	10	30	31	< 0.001
Saprophytic	41	27	29	0.021
Negative-culture	9	3	0	0.004

Data are number of patients

CNS coagulase-negative staphylococci, *CPS* coagulase-positive staphylococci

*The sum of patients by group of microorganisms exceeds 60 patients, since more than one type of microorganism may be isolated from a single sample

† Similarly, various species of the same type of flora (pathogenic or saprophytic) may exist in one sample, so the sum of pathogens or saprophytes groups may not coincide with the total samples classified by type of flora. However, the sum by type of flora results in the total of patients in each column due to each sample being classified as pathogenic, saprophytic or negative-culture

‡ Pearson's chi-square test

Similarly, samples from patients with lacrimal obstruction had a higher MD in anophthalmic socket, both in the pre-prosthesis (0.637 ± 0.183 vs. 0.339 ± 0.251, $P = 0.032$) and in the retro-prosthesis samples (0.807 ± 0.129 vs. 0.379 ± 0.236, $P = 0.002$). The use of medications in the socket was related to modifications of flora in the pre-prosthesis samples. A higher MD was determined in patients who used self-prescribed antibiotics (0.553 ± 0.251 vs. 0.302 ± 0.231, $P = 0.014$) and a greater rate of coryneform bacteria in those who employed lubricants (2/11 vs. 0/49, $P = 0.035$).

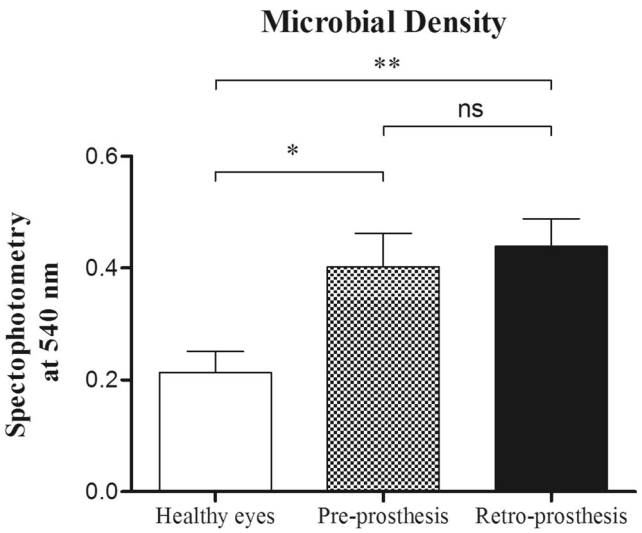

Fig. 1 Microbial density measured by spectrophotometry at 540 nm in 29 randomly selected patients. Analysis of variance showed a statistical difference ($P = 0.004$) that posteriorly was studied by Bonferroni's multiple comparison test, revealing a lower microbial density in healthy eyes compared to anophthalmic socket samples. ns = no statistical difference. *$P = 0.027$. **$P = 0.006$

Frequent manipulators showed a higher MD in the pre-prosthesis samples (0.468 ± 0.263 vs. 0.186 ± 0.113, $P < 0.001$), without any statistical association by groups of microbial flora.

Gender, type of surgery, implant size (categorized into two groups: diameter of 20 mm or greater, and 18 mm or less), and longevity of the artificial eye showed no influence on the flora, either in healthy eyes or in sockets.

Influence of the conjunctival flora and other factors on the grade of comfort of the socket

The results of the relationship between socket flora and GCS are shown in Table 3. Although GCS was mainly related to retro-prosthesis flora, pathogenic microorganisms were associated with worse comfort in both the pre-prosthesis ($P = 0.026$) and retro-prosthesis ($P = 0.002$) samples. When

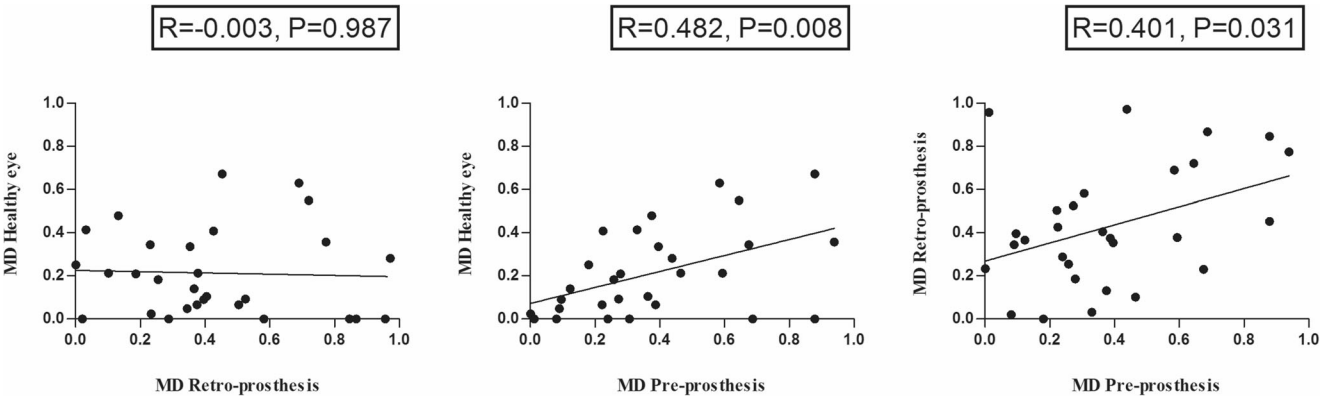

Fig. 2 Correlations of microbial density measured by spectrophotometry (MD) at 540 nm between samples from different conjunctival locations, in 29 randomly selected patients. Note the positive correlations of pre-prosthesis MD with retro-prosthesis MD and healthy eyes MD

the flora was studied by groups of microorganisms, Gram-negative bacteria in the retro-prosthesis samples were related to poor GCS ($P = 0.008$). However, this association was not observed in pre-prosthesis samples ($P = 0.225$). The only group of microorganisms linked to good GCS was the one formed by other CNS, excluding *S. epidermidis*, in the retro-prosthesis samples ($P = 0.030$).

Regarding the MD (Table 4), it was significantly higher ($P = 0.017$) in the pre-prosthesis samples of patients with poor GCS (0.663 ± 0.250), compared to those with good (0.289 ± 0.196) or fair (0.359 ± 0.263) GCS. In the retro-prosthesis samples, the MD was also higher in patients with poor GCS (0.640 ± 0.319), although this difference was not statistically significant ($P = 0.123$). The MD from healthy eyes samples was not related to GCS (Table 4).

Besides the flora, the relationship between GCS and the variables collected in the clinical examination of patients (see Methods) was assessed. The most interesting findings were obtained from variables in relation to the artificial eye care and the characteristics of the socket. A lesser frequency of prosthetic eye removal ($P = 0.031$) and absence of mucus ($P = 0.034$) or hyperemia ($P = 0.014$) in the socket were associated with good GCS (Table 5). The remaining clinical features, including absence of an orbital implant ($P = 0.549$) or use of antibiotic eyedrops in the socket ($P = 0.110$), were not related to changes in GCS.

Table 2 Patient characteristics associated with increased streptococci and pathogenic flora in the anophthalmic socket

	Pre-prosthesis				Retro-prosthesis			
	Streptococci		Pathogenic flora		Streptococci		Pathogenic flora	
	($n = 14$)	*P* value*	($n = 30$)	*P* value*	($n = 13$)	*P* value*	($n = 31$)	*P* value*
Anophthalmic side								
Right ($n = 24$)	2	0.025	8	0.051	5	0.898	10	0.206
Left ($n = 36$)	12		22		8		21	
Systemic medication								
Yes ($n = 44$)	9	0.597	22	0.804	6	0.032	23	0.876
No ($n = 16$)	5		8		7		8	
History of endophthalmitis								
Yes ($n = 16$)	6	0.223	8	0.949	7	0.032	11	0.110
No ($n = 44$)	8		22		6		20	
Date of surgery								
20 years ago or less ($n = 38$)	3	<0.001	14	0.016	6	0.260	17	0.158
Over 20 years ago ($n = 22$)	11		16		7		14	
Orbital implant								
Yes ($n = 33$)	3	0.004	11	0.005	4	0.047	13	0.035
No ($n = 27$)	11		19		9		18	

Data are number of patients

*Pearson's chi-square test

Table 3 Conjunctival flora in pre-prosthesis and retro-prosthesis socket samples* by grade of comfort with the artificial eye

Conjunctival flora determinations	Pre-prosthesis				Retro-prosthesis			
	GCS				GCS			
	Good (n = 32)	Fair (n = 18)	Poor (n = 10)	P Value†	Good (n = 32)	Fair (n = 18)	Poor (n = 10)	P Value†
Microorganism groups								
S. epidermidis	21	15	7	0.407	22	14	6	0.601
Others CNS	7	1	0	0.105	11	2	0	0.030
CPS	5	2	4	0.141	5	2	4	0.141
Coryneform bacteria	2	0	0	0.404	0	2	0	0.089
Micrococcus spp	1	0	0	0.641	0	0	0	–
Streptococci	10	2	2	0.261	9	1	3	0.139
Enterococci	1	1	0	0.732	0	1	0	0.305
Gram-negative bacteria	6	1	3	0.225	3	2	5	0.008
Fungi	0	1	0	0.305	0	1	0	0.305
Type of flora								
Pathogenic	17	5	8	0.026	15	6	10	0.002
Saprophytic	13	12	2	0.045	17	12	0	0.002
Negative cultures	2	1	0	0.725	0	0	0	–

Data are number of patients

GCS Grade of comfort of the socket

*Similarly to Table 1, the sum of patients in each group of microorganisms exceeds the total number of samples, since more than one type of microorganism may be isolated from a single sample. Likewise, the sum of pathogenic or saprophytic microorganism groups may not coincide with the number of samples by type of flora

†Pearson's chi-square test

Discussion

One of the most common complaints of anophthalmic patients is socket discomfort. However, socket discomfort encompasses a broad spectrum of signs and symptoms. While some patients say that they do not notice that they are using an artificial eye, others complain of daily discomfort and discharge. These annoyances are usually blamed on a scratched or poorly fitting prosthesis, inadequate care of the prosthesis, or an allergy to the material from which it is made. However, conjunctival flora could be involved in the discomfort, as is the case with other epithelia [10–15]. Therefore, the study of conjunctival microbiota in anophthalmic patients may provide a new approach to improving socket discomfort.

This study has determined that the composition of the flora of anophthalmic sockets differed from the microflora of

Table 4 Microbial density* by Grade of comfort with the artificial eye, according to location of samples

	Grade of comfort with the artificial eye			P Value†
	Good (n = 13 patients)	Fair (n = 11 patients)	Poor (n = 5 patients)	
Location of samples				
Healthy eyes	0.230 ± 0.205	0.195 ± 0.225	0.210 ± 0.165	0.920
Pre-prosthesis socket	0.289 ± 0.196	0.359 ± 0.263	0.663 ± 0.250	0.017
Retro-prosthesis socket	0.440 ± 0.240	0.344 ± 0.248	0.640 ± 0.319	0.123

Data are mean ± standard deviation unless otherwise indicated

*Microbial density was measured by spectrophotometry at 540 nm in only 29 patients (see Microbial Density Assessment in Methods section)

† Analysis of variance

Table 5 Relationship between grade of comfort with the artificial eye and clinical features of ocular prostheses and sockets

Characteristics	Grade of comfort with the artificial eye			*P* Value*
	Good (n = 32)	Fair (n = 18)	Poor (n = 10)	
Ocular prosthesis				
Longevity less than 1 year	15	7	2	0.316
Removal once a week or more	12	13	7	0.031
Socket				
Trauma history	16	9	4	0.846
Endophthalmitis history	8	6	2	0.711
Evisceration surgery	28	17	10	0.403
Absence of orbital implant	14	7	6	0.549
Epiphora	5	5	4	0.245
Lacrimal pathway obstruction	4	1	3	0.186
Absence of abnormal mucus	22	7	3	0.034
Conjunctival hyperemia	4	8	5	0.014
Use of antibiotic drops	2	4	3	0.110

Data are number of patients

*Pearson's chi-square test

healthy eyes. Sockets presented increased pathogens (Table 1) and a higher MD (Fig. 1). This evaluation accords with previous reports that described an increase of pathogens in sockets (Gram-negative bacteria [5, 6, 8], anaerobes [5, 16], CPS [8], and streptococci [8]), despite the fact that we observed only a higher rate of Gram-negative bacteria and streptococci (Table 1).

We have also quantified the whole conjunctival flora by measuring the MD using spectrophotometry. Although microorganisms of the conjunctival flora may have different cell sizes and growth rates (factors influencing the optical density measurement), spectrophotometry provides an adequate correlation between optical density and the number of microorganisms per volume in the sample [28]. This study has shown that the handling required for the measurement of optical density does not alter the obtained conjunctival flora. Hence, we think that this technique should be used routinely in clinical studies of conjunctival flora.

The analysis of the MD data identified a positive correlation between the MD of conjunctival samples obtained from the pre-prosthetic and retro-prosthetic spaces of the socket and, even more interestingly, between the pre-prosthetic space and the healthy eye (Fig. 2). This relationship, in which the anophthalmic socket may act as a reservoir of microorganisms, had already been suggested by other authors [8, 22, 23], and it explains why some studies found no differences between sockets and fellow eyes [22, 23]. Lopez et al. [23] described a similar rate of pathogens in sockets and fellow eyes (37% vs. 27%), but a significantly lower one in the control group (8%), made up of healthy eyes of non-anophthalmic patients. Similarly, our outcomes in healthy eyes flora showed a coryneform bacteria rate of 5% (Table 1) when it is usually over 25% in non-anophthalmic patients [17, 19, 21]. Since coryneform bacteria rarely cause endophthalmitis [29], a relative increase in more pathogenic bacteria exists in the fellow eyes of these patients.

Our results therefore indicate that the flora of healthy eyes approaches the normal conjunctival flora, with a predominance of saprophytic species such as *S. epidermidis*. However, the correlation between the flora of the sockets and healthy eyes (Fig. 2) and the decrease in some saprophytic species such as coryneform bacteria suggest a certain degree of dysbiosis in the conjunctiva of the eye healthy as well. These findings confirm the need for the special anti-infection care during intraocular surgery in anophthalmic patients, as recommended in prior studies [8, 22, 23].

Conversely, anophthalmic socket flora has an obvious dysbiosis, with increased bacterial density and rate of pathogens. This suggests that the presence and manipulation of the ocular prosthesis are the factors responsible for the microbial imbalance existing in the conjunctival flora of the socket. Nevertheless, other clinical and demographic characteristics of the patients were associated with socket dysbiosis. The absence of an orbital implant was the main factor related to increased pathogenic flora on the socket (Supplemental Material 2). Despite orbital implants providing several benefits in anophthalmic patients [30, 31], there are no studies in the literature that evaluate their effect on the flora of the socket. The dead space between the posterior surface of the prosthesis and the anterior surface of the socket is probably larger in the case of

an absence of an orbital implant, so it seems reasonable that microorganisms can accumulate and grow [6, 25].

Regarding other variables better known as modifying factors of conjunctival flora, mucus and hyperemia in the socket have been associated with an increase of pathogens [5]. However, in our study this effect was not observed. Instead, inflammation of the socket was linked to changes in the healthy eye flora (more pathogens if there was hyperemia in the socket and higher MD if there was excessive mucus). These results strengthen the hypothesis that microorganisms from the anophthalmic socket are transferred to the fellow eye.

The higher MD obtained in the socket of patients with epiphora and lacrimal obstruction is consistent with previous reports [32, 33], although there was no increase of pathogens in our samples. Topical antibiotics reduce the conjunctival flora during the first hour after their application [34], but chronic use cause a selection of resistant strains (especially *S. epidermidis*) and promote their growth [35]. Furthermore, they have not been associated with a decrease of discharge in anophthalmic sockets [3]. In agreement with these reports, we related antibiotics to a higher MD in pre-prosthesis samples (0.553 ± 0.251 vs. 0.302 ± 0.231, $P = 0.014$) but not to the pathogens rate. Based on these outcomes we propose a punctual use of topical antibiotics for 7–10 days to treat acute bacterial conjunctivitis and avoiding its continuous use in patients with chronic discharge. Although some disinfectants like povidone may have a broader antimicrobial spectrum, they are generally poorly tolerated. Thus, in case of socket discomfort, other factors should be assessed such as the prosthesis surface, its adaptation to the socket, the absence of orbital implant, the frequency of prosthetic eye removal, or the permeability of the lacrimal pathway.

The key finding in this study is that conjunctival flora of the socket is related to GCS. Although the relationship between conjunctival flora and discomfort in the socket has been evaluated by other studies [5–9], these have pointed in both directions. We found that patients who reported poor GCS showed increased pathogenic microorganisms in both pre-prosthesis and retro-prosthesis spaces (Table 3) and higher MD (0.663 ± 0.250) in the pre-prosthesis space (Table 4). Moreover, after performing an analysis by groups of microorganisms in retro-prosthesis samples, Gram-negative bacteria were associated with poor GCS, whereas a higher rate of CNS (excluding *S. epidermidis*) were related with good GCS (Table 3). In order to promote the growth of these beneficial species, probiotic or prebiotic eye drops may be employed. The direct use of a cultured broth with live microorganisms (probiotic eye drops) presents problems in preservation, dosage, and microbial contamination. These disadvantages can be avoided by using prebiotic substances, since some of them only increase the native saprophytic flora. We have proved in vitro anti-biofilm activity of some non-toxic plant metabolites (genistein, protocatechuic acid, cranberry extract, p-hydroxybenzoic acid and resveratrol) against *S. aureus* but not against *S. epidermidis* in which, surprisingly, these metabolites stimulated the biofilm formation [36]. However, the microbial activity and clinical repercussion of these substances in the anophthalmic sockets remains unknown.

Regarding previous studies, a high proportion of pathogens in symptomatic patients have been documented by Christensen and Fahmy [5] (54% vs. 17%, $P = 0.013$), but these were unrelated to any specific group of microorganisms. Thygeson and Kimura [9] also found a high pathogens rate in symptomatic sockets (87%), although the rate in asymptomatic patients was not indicated in this study. On the other hand, Vasquez et al. [6] have not determined changes in the flora of symptomatic sockets, but they did not group the microorganisms according to pathogenicity. Likewise, Miller et al. [8] did not obtain a difference in the socket pathogens rate between symptomatic and asymptomatic patients (87% vs. 67%, $P = 0.114$), but viridans group streptococci were considered saprophytic flora, whereas they have been classified as pathogens in our study and in others [5, 9]. *Streptococcus viridans* species are the second-most frequently identified cause of endophthalmitis, behind only *S. epidermidis* [29]. However, they account for less than 5% of the isolates in the conjunctival flora [17, 19, 21], so we think that this species should be classified as a pathogen. Therefore, grouping of microorganisms on the basis of their pathogenicity allows a heightened power of analysis, but outcomes may vary according to the selected species. To establish an objective classification of the pathogenicity for a microorganisms group, we recommend comparing the isolation rates from healthy conjunctival flora and from intraocular samples of endophthalmitis.

Besides the conjunctival flora, other clinical data of patients (abnormal mucus or hyperemia in socket and prosthesis removal regime) were linked to GCS (Table 5). The absence of abnormal mucus or hyperemia in the sockets was associated with good comfort. This relationship is logical, since hyperemia and excess mucus in the socket were associated with each other in our study ($P < 0.001$) and discharge was one of the criteria used to define GCS.

More interestingly, there was an association between the frequency of prosthetic eye removal and GCS. We noted a worsening of symptoms in patients with weekly cleaning regimes, as Pine et al. [3] had previously reported. There are several theories as to the relationship between frequency of prosthesis removal and discharge from the socket. On one hand, asymptomatic patients have less need to remove the artificial eye, which decreases the mechanical trauma to the conjunctiva [37, 38]. On the other hand, the prosthetic eye is covered with mucoprotein deposits when it is used continuously [39]. When the mucoproteins are attached at the posterior surface of the artificial eye, rather than producing inflammation they prevent it, probably by improving the lubrication

of the prosthesis [40]. This explains why repolishing regimes have a limited impact on experience of discharge [3, 6]. Moreover, socket microflora was also related to frequency of prosthesis removal. Vasquez and Linberg [6] found that patients who frequently manipulated their prosthesis had a significantly higher proportion of Gram-negative bacteria in their sockets, although other authors did not observe this association [5, 23]. We noted a higher MD only in the pre-prosthesis samples of frequent manipulators (0.468 ± 0.263 vs. 0.186 ± 0.113, $P < 0.001$), with no association with any specific group of microorganisms. Although this triple association found in our study (frequent prosthesis removal—high MD in pre-prosthesis space—poor GCS) does not indicate the direction of cause and effect, frequent prosthesis removal should be avoided. Patients should be advised that they must permanently wear their ocular prosthesis for at least a week.

Study limitations

We did not find in the literature a validated or commonly applied scale to determine the GCS in anophthalmic patients, so we have had to use a questionnaire designed by ourselves. Our isolation technique failed in obtaining anaerobes. Therefore, their possible involvement in GCS remains unknown. The initial pre-culture in conventional BHI under aerobic conditions probably prevented their further isolation. This situation could be resolved by using pre-reduced BHI supplemented with hemin and vitamin K [16] or thioglycollate broth.

Despite having observed a relationship between flora and GCS, determining the etiological role of microorganisms in GCS is impossible with the design of our study.

These findings indicate the need for a prospective, longitudinal study of anophthalmic patients to describe how symptoms and flora change over time and to determine if the modification in microbial composition is the cause of symptoms or a consequence of manipulations in the anophthalmic socket.

Conclusion

The socket in anophthalmic patients shows a conjunctival dysbiosis that affects the flora of the fellow eye. Decreased saprophytic species in fellow eyes could promote the development of post-surgical endophthalmitis. Therefore, we recommend extreme anti-infection care during intraocular surgery on the fellow eye of an anophthalmic patient.

The feeling of discomfort in the socket of anophthalmic patients is very common, multicausal, and difficult to resolve. Increased pathogenic microorganisms in the socket are associated with poor GCS, so actions to resolve this dysbiosis may be useful in improving comfort. Chronic use of antibiotics is not related to decreased pathogens or an improvement in GCS, so we recommend avoiding long regimes of topical antibiotics. Some treatments such as lubricants, prebiotics, or probiotics may help to increase conjunctival saprophytic species in the socket and improve the socket symptoms. In this sense, we are conducting more studies in this area.

Finally, frequent removal of the prosthesis was associated with discomfort and increased MD in the socket. Therefore, we recommend decreases in the removal frequency of the artificial eye in patients with discomfort and who handle their prosthesis frequently.

BHI, brain heart infusion; CNS, coagulase-negative staphylococci; CPS, coagulase-positive staphylococci; GCS, grade of comfort of the socket; MD, microbial density; NS, no statistical difference.

Compliance with ethical standards

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Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

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