Comparing the Effects of Two Different Irrigation Solutions on an Isolated Perfused Vertebrate Retina

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Key Words

Electrophysiology · Toxicity · Vitreoretinal surgery ·
Intraocular drugs

Abstract

Purpose: To compare the effect of a taurine-containing intraocular irrigation solution (PuriProtect™) to a standard irrigation solution (BSS™) we evaluated the retinal function using an electroretinogram (ERG) and analyzed the survival of retinal ganglion cells on isolated whole mount retinas. **Materials and Methods:** During ERG recordings, each irrigation solution was superfused for 45 min with the relevant irrigation solution. To investigate the effects on photoreceptor function, 1 mM aspartate was added to obtain a-waves. The recovery of the a- and b-wave was monitored after superfusing the retinas with standard medium again. To evaluate the percentage of dead ganglion cells, retinas were stored for 24 h at 4°C in darkness and after staining the retinas with ethidium homodimer-1 the retinas were analyzed using fluorescence microscopy. **Results:** The application of standard medium supplemented with 2 mM taurine resulted in a significant increase of the b-wave amplitude compared to standard medium alone. The a-wave amplitudes showed no significant changes under taurine supplementation. Compared to standard medium BSS showed no significant

decrease in b-wave amplitudes, but a significant decrease in a-wave amplitudes. In contrast to BSS there were no significant changes in the a- or b-wave amplitudes detectable after the application of PuriProtect. At the end of the washout period no significant changes in a- or b-wave amplitudes were recorded for any tested irrigation solution. Retinas stored for 24 h in PuriProtect or in standard medium with taurine had a statistically significant smaller amount of dead cells than retinas stored in standard medium without taurine supplementation. **Conclusions:** BSS does not seem to be an ideal irrigation solution, because it compromises the a-wave in the ERG. In contrast to BSS, PuriProtect showed no significant impact on the ERG and showed a better long-term effect on ganglion cell survival. Taurine supplementation, therefore, seems to be neuroprotective and its supplementation to an intraocular irrigation solution favorable for the retina.

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Introduction

During vitreoretinal surgery many factors that may be harmful to the retina must be considered. Endoillumination [1], continuous flow of an irrigation solution to maintain inner pressure during and after vitrectomy [2], the

mechanical manipulation of the retina up to an artificial detachment and the suppression of blood flow reducing the nutrition of neuronal tissue have been shown to have a negative effect on the retina [3–5]. The fact alone that surgery on the retina is necessary indicates in most cases that the retina is already damaged in morphology and function. Not many factors can be altered or optimized in order to reduce unnecessary iatrogenic trauma on the retina. Irrigation solutions, however, are one area that can be changed. They remain in the posterior chamber of the eye for up to 48 h after vitreoretinal procedures [6] and have a direct effect on the retina during the procedure and for a prolonged interval after surgery. In order to minimize negative effects or even to obtain a protective effect on neuronal tissue, new irrigation solutions have been developed since the introduction of balanced salt solution (BSSTM) in 1960. Scientists have tried to optimize chemical composition to reduce iatrogenic trauma by adding substances with neuroprotective effects.

Taurine (C₂H₇NO₃S) is a β -amino acid that can be found in high concentrations in many tissues including the mammalian heart, liver, central nervous system and retina [7, 8]. It is believed to be involved in cell volume homeostasis, antioxidant defense, protein stabilization, cell development and stress responses [9, 10]. The latter has been of particular interest to recent investigations after researchers have found neuroprotective effects of taurine in cell death signaling mechanisms. Taurine was also beneficial after cerebral ischemia in animal models and protected cells against excitotoxicity in cell cultures [11, 12]. Pasantes-Morales and Cruz [13] have shown that in several species the retina has a high content of taurine. Lücke et al. [14] demonstrated the protective effect of taurine against a toxic agent and in the same study measurements were also taken of an isolated, bovine, perfused retina [14]. Following this study a new intraocular irrigation solution – PuriProtectTM – was formulated, which contains the same components as BSS, with the addition of 3 mM taurine.

Besides the newly introduced PuriProtect another irrigation solution (BSS PlusTM) was developed with the goal of adding neuroprotective agents. BSS has been the standard intraocular irrigation solution since the 1960s. Although many studies have shown the disadvantages of BSS and many scientists have pointed out that it does not meet the requirements for an ideal intraocular surgical irrigation solution, it is still widely used [6].

The aim of this study was to evaluate and compare the effects of BSS solution with PuriProtect. We evaluated the short-term effect of the irrigation solutions on the retina,

focussing on their functionality using the electroretinogram (ERG) and their long-term effect by evaluating retinal ganglion cell survival with histological staining techniques.

Materials and Methods

Aspartate, glucose and other chemicals were obtained from Merck (Merck Pharma GmbH, Darmstadt, Germany) at proanalysis grade. The commercially available irrigation solutions BSS (AMO[®], Groningen, The Netherlands) containing 155.7 mM NaCl, 10.1 mM KCl, 3.3 mM CaCl₂, 1.5 mM MgCl₂ and 29 mM C₂H₃NaO₂ with a final pH of 7.5 and an osmolality of 305 mosm/kg and PuriProtect (Zeiss[®] GmbH, Jena, Germany) containing 155 mM NaCl, 10.1 mM KCl, 3 mM CaCl₂, 1.5 mM MgCl₂, 29 mM C₂H₃NaO₂ and 2.8–3.2 mM C₂H₇NO₃S with a final pH of 7.4 and an osmolality of 290 mosm/kg were donated by Zeiss. We added 5 mM glucose to each irrigation solution to guarantee a stable ERG recording [15]. Live/DeadTM viability/cytotoxicity assay (L-7013) for mammalian cells containing ethidium homodimer-1 was purchased from Molecular Probes (Eugene, Oreg., USA).

Methods

Bovine eyes were obtained and prepared within 15 min post-mortem and were transported in darkness in a serum-free standard medium containing 120 mM NaCl, 2 mM KCl, 0.1 mM MgCl₂, 0.15 mM CaCl₂, 1.5 mM NaH₂PO₄, 13.5 mM Na₂HPO₄ and 5 mM glucose with a final pH of 7.8. The preparation was performed as described recently [16]. The ERG was recorded via two silver/silver-chloride electrodes on either side of the retina. The recording chamber containing a piece of retina was placed in an electrically and optically insulated box. A roller pump kept the perfusion velocity at 1 ml/min, while the temperature was kept constant at 30°C. The perfusion medium was pre-equilibrated and saturated with oxygen [17]. After the retina was dark-adapted for 1 h at 30°C under constant perfusion the ERG was elicited at intervals of 5 min using a single white xenon flash for stimulation. A flash intensity of 6.3 mlx at the retinal surface using calibrated neutral density filters (Kodak Wratten filter) and a duration of light stimulation of 10 ms controlled by a timer (Photopic Stimulator PS33 Plus; Grass, Warwick, R.I., USA) were set. The ERG was filtered and amplified (100-Hz high filter, 50-Hz notch filter, 100,000 $\times$ amplification) using a Grass RPS312RM amplifier. With an analog-to-digital data acquisition board (PCI-MIO-16XE-50; National Instruments, Austin, Tex., USA) the data were processed and converted in a desktop computer (PC compatible).

Electrophysiology

For the evaluation of the b-wave the retina was superfused with the standard medium and stimulated repeatedly until stable b-wave amplitudes for five consecutive measurements were recorded. This b-wave acted as baseline measurement. Thereafter the irrigation solution of interest ($n = 5$, for each irrigation solution) was applied, and responses were recorded for 45 min. After the application period of 45 min retinas were reperfused with the standard medium for 75 min (washout period) and the changes of the b-wave amplitudes were recorded. The b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave.

Fig. 1. The ERG from the isolated perfused bovine retina. **a** The b-wave is dominant in the ERG of the isolated perfused bovine retina under scotopic light conditions. It results from a 10-ms light stimulus at a light intensity of 6.3 mlx at scotopic lighting conditions, which is marked by the arrow. **b** The a-wave is dominant in the ERG of the isolated perfused bovine retina after blocking the b-wave by adding 1 mM aspartate to the nutrient solution. The a-wave was generated by using a 10-ms light stimulus of 6.3 mlx at scotopic lighting conditions.

To investigate the effect of the different irrigation solutions on the a-wave the b-wave was suppressed adding 1 mM aspartate to the standard medium. After the blocking of the b-wave the a-wave represents the photoreceptor potential. The a-wave was then recorded under scotopic conditions with a flash intensity of 6.3 mlx. Aspartate is an inhibitor of the synaptic transmission at the level of the first retinal synapse and thereby abolishes the b-wave and unmasks the photoreceptor potential P III. The supplementation of 1 mM aspartate was kept throughout the whole experiment. Under these conditions, the influence of the different irrigation solutions on the photoreceptors was analyzed. After recording a stable photoreceptor potential for 30 min (baseline measurement) the retinas were exposed for 45 min to each irrigation solution followed by a washout period of 75 min in which the retinas were superfused with the standard medium again.

A series of 5 independent experiments on different retinas was conducted for each irrigation solution tested. The percentage reduction of the a- and b-wave amplitudes to the baseline measurement was calculated for each irrigation solution. The recovery of the a- and b-wave at the end of the washout was compared to the corresponding baseline amplitude of the a- and b-wave before the perfusion with the different irrigation solutions.

Live/Dead Assay

In order to investigate a possible long-term effect of the different irrigation solutions, bovine retinas were stored in BSS, PuriProtect or standard medium for 24 h in 6 different petri dishes at 4°C protected from light. Afterwards the Live/Dead viability/cytotoxicity assay (L-7013) for mammalian cells containing ethidium homodimer-1 was performed without using calcein according to the manufacturer's instructions. Six corresponding regions from the central area and the central periphery from each retina

embedded in a different irrigation solution were chosen at 200× magnification and dead cells counted using a fluorescence microscope (Axiovert 200, magnification ×40 and Axiovision imaging; Zeiss). To facilitate cell counting a special imaging software program was used (AnalySIS®; Soft Imaging System GmbH, Münster, Germany). After 24 h at 4°C the dead ganglion cells were counted and the percentage of dead cells in the retinal ganglion cell layer was calculated for each irrigation solution and compared to the standard medium.

Statistics

For statistical analysis the software 'Origin 6.0' (Microcal) was used. Data were calculated throughout as the mean with standard deviation. Significance was estimated by the Student t test and p values <0.05 were considered statistically significant.

Results

Environmental parameters such as pH, osmotic pressure, temperature and pO₂ remained unchanged during all tests. After reaching stable b-wave amplitudes (fig. 1a), each intraocular irrigation solution was applied. While testing PuriProtect supplemented with 5 mM glucose an increase of 5.4%, which did not reach statistical significance ($p > 0.05$), was seen at the end of the application period. After the washout period no change in b-wave recordings could be detected (fig. 2a). During perfusion with BSS supplemented with 5 mM glucose a statistically

Fig. 2. Effects of the irrigation solution PuriProtect supplemented with 5 mM glucose (**a**), BSS supplemented with 5 mM glucose (**b**) and standard medium supplemented with 2 mM taurine (**c**) on the b-wave amplitude of the ERG in the model of the isolated, perfused, bovine retina. Average of representative drug series (number of experiments, $n = 5$). The horizontal bar above the curve marks the application. The curves are depicted as mean $\pm$ SD. Three representative SDs for each drug series are given.

nonsignificant ($p = 0.12$) reduction in the b-wave amplitude of 21.3% could be observed. At the end of the wash-out of BSS a nonsignificant ($p > 0.05$) increase of 6.3% was noted (fig. 2b). Interestingly, when the standard solution with 2 mM taurine was tested, a statistically significant ($p < 0.01$) increase of the b-wave amplitude of 41.7% was recorded. At the end of the washout period a nonsignificant decrease of 8.3% was measured (fig. 2c). Original values are shown in table 1.

During the perfusion studies testing only the effects on the P III component, a statistically nonsignificant ($p > 0.05$) reduction of 20.83% was noted during the application of PuriProtect supplemented with 5 mM glucose. At the end of the washout no significant changes in a-wave amplitudes could be detected (reduction of 2.1%; $p > 0.05$; fig. 3a). Testing BSS and 5 mM glucose a statisti-

cally significant ($p < 0.001$) decrease of the a-wave amplitude of 51.0% was recorded. Full recovery of a-wave amplitudes at the end of the washout was noted (fig. 3b). During the perfusion with the standard solution and 2 mM taurine the a-wave amplitude showed a statistically significant ($p > 0.05$) reduction of 3.8%, followed by a full recovery at the end of the washout (fig. 3c). Original values are shown in table 2.

Live/Dead Assay

To investigate a long-term effect of the irrigation solutions on the survival of retinal ganglion cells, the Live/Dead viability/cytotoxicity assay (L-7013) was used to stain dead ganglion cells (fig. 4). Ganglion cells were morphologically identified and the ratio between dead ganglion cells and ganglion cells was calculated. The per-

Table 1. Mean and SD of the ERG b-wave amplitudes

Time min	Standard solution with 2 mM taurine		BSS with 5 mM glucose		PuriProtect with 5 mM glucose	
	b-wave amplitude mean, μ V	SD, μ V	b-wave amplitude mean, μ V	SD, μ V	b-wave amplitude mean, μ V	SD μ V
0	7.4	0.55	10.0	0.00	6.8	0.84
5	7.4	0.55	9.6	0.55	7.2	1.30
10	7.4	0.55	10.2	0.84	6.8	1.10
15	7.4	0.55	10.2	0.84	7.2	1.10
20	7.4	0.55	9.8	0.84	7.4	0.55
25	7.2	0.45	9.4	0.89	7.4	1.14
30	7.8	0.84	10.0	0.71	6.4	0.90
35	8.4	1.67	9.4	0.55	7.8	1.79
40	8.8	1.48	9.4	0.89	8.0	2.00
45	10.0	1.22	8.4	2.61	7.4	2.19
50	10.6	1.52	8.8	1.30	8.0	2.00
55	10.4	1.14	8.2	1.92	8.0	1.73
60	9.8	0.84	7.4	2.51	8.8	3.70
65	10.6	0.89	7.8	2.39	7.6	1.95
70	10.2	0.45	7.0	2.92	7.8	2.17
75	10.6	1.34	7.6	2.70	7.4	1.14
80	10.6	0.89	8.4	2.70	6.8	1.10
85	9.2	0.84	9.0	1.87	6.8	1.92
90	8.2	0.84	8.6	1.52	6.8	0.45
95	8.4	1.14	9.0	1.22	6.2	0.45
100	7.8	1.30	8.0	2.24	6.0	1.22
105	7.4	0.55	8.8	1.30	6.4	0.55
110	7.6	0.89	8.6	1.67	6.4	0.55
115	7.2	0.45	9.2	0.84	7.8	1.30
120	7.6	0.89	8.6	1.14	7.2	0.84
125	7.4	0.55	9.0	1.22	7.2	0.84
130	7.6	0.89	9.6	1.14	6.8	1.10
135	7.4	0.55	9.2	0.84	6.8	1.10
140	7.4	0.55	9.0	1.22	6.4	0.55
145	7.2	0.45	10.0	2.00	7.4	1.14

Table 2. Mean and SD of the ERG a-wave amplitudes

Time min	Standard solution with 2 mM taurine		BSS with 5 mM glucose		PuriProtect with 5 mM glucose	
	a-wave amplitude mean, μ V	SD, μ V	a-wave amplitude mean, μ V	SD, μ V	a-wave amplitude mean, μ V	SD μ V
0	-10.0	0.00	-9.8	0.45	-10.0	0.00
5	-10.6	0.55	-9.6	0.55	-10.0	0.71
10	-10.6	0.55	-9.8	0.45	-9.8	0.45
15	-10.4	0.89	-9.8	0.45	-9.4	0.89
20	-10.2	0.45	-9.8	0.45	-10.0	0.71
25	-10.6	0.89	-9.8	0.45	-9.6	1.14
30	-10.4	1.34	-9.4	0.90	-9.4	0.89
35	-9.8	1.10	-8.8	1.30	-9.2	1.10
40	-10.6	0.89	-8.4	2.07	-8.4	0.90
45	-10.4	1.14	-8.0	1.58	-8.8	1.10
50	-10.8	1.79	-8.0	1.87	-8.2	1.92
55	-10.4	2.30	-6.8	1.48	-8.0	2.00
60	-10.2	1.48	-6.0	1.41	-9.2	0.84
65	-10.4	1.82	-5.4	0.55	-8.0	1.41
70	-10.2	1.92	-4.8	0.84	-7.6	2.51
75	-10.2	1.48	-5.0	0.71	-7.8	2.28
80	-10.2	1.92	-5.0	0.71	-8.4	2.30
85	-9.6	1.67	-4.8	0.84	-8.0	2.74
90	-9.8	1.48	-5.8	2.17	-8.2	2.68
95	-10.2	1.30	-7.4	3.65	-8.6	2.79
100	-10.0	1.58	-8.6	3.05	-8.8	2.49
105	-10.4	1.82	-9.4	2.51	-9.2	1.92
110	-10.0	1.58	-9.6	2.07	-8.8	2.28
115	-10.0	1.87	-10.0	1.58	-9.8	1.92
120	-10.2	1.64	-10.0	1.87	-9.6	1.52
125	-10.2	1.48	-9.6	1.82	-9.4	1.82
130	-10.6	2.07	-10.0	1.41	-9.4	1.34
135	-10.2	1.92	-9.6	1.82	-9.0	1.58
140	-10.4	1.82	-9.8	2.05	-9.2	1.64
145	-10.6	2.30	-9.8	1.30	-9.4	1.82

centage of dead ganglion cells in retinas stored in PuriProtect was 30.1% ($\pm$ SD 14.1), but in retinas stored in BSS it was 84.0% ($\pm$ SD 12.4). In the control group (standard medium) 69.6% ($\pm$ SD 3.9) of the ganglion cells were dead and in the standard medium supplemented with 2 mM taurine 36.3% ($\pm$ SD 13.9). All solutions were preoxygenated before storage. Only the retinas that were stored for 24 h in PuriProtect or in the control medium supplemented with 2 mM taurine had a statistically significant smaller percentage of dead ganglion cells compared to the control group ($p = 0.0014$ and $p = 0.004$, respectively; table 3).

Discussion

The isolated vertebrate retina is a highly sensitive and standardized tool to investigate the biocompatibility of agents in direct contact with the retina [17, 18]. It shows similar responses to human retinas [19]. Moreover, it is possible to examine the isolated effect of an intraocular solution on the retina alone in a controlled environment as all parameters are more securely controlled than in any in vivo animal model [16]. High implicit times result from the experimental setup. We added 5 mM glucose to BSS and PuriProtect during our tests because metabolism in the isolated retina depends primarily on glucose [15]. In the in vivo situation glucose would be delivered

Fig. 3. The graph shows the effects of the irrigation solution Pu-riProtect supplemented with 5 mM glucose (a), BSS supplemented with 5 mM glucose (b) and standard medium supplemented with 2 mM taurine (c) on the a-wave amplitude of the ERG in the model of the isolated, perfused, bovine retina. Average of representative drug series (number of experiments, $n = 5$). The black bar marks the application. Three representative SDs for each drug series are given. The dotted line marks the application of aspartate (1 mM) to unmask photoreceptor potential.

by the retinal blood vessels. Nevertheless, a lack of glucose in a surgical irrigation solution could be regarded as critical in situations with diminished retinal blood flow. In this *in vitro* scenario we tried to study the biocompatibility of each irrigation solution alone and not their metabolic entities. Using the above-mentioned organ culture method of the isolated vertebrate retina we could show that there were no statistically significant negative effects of the irrigation solutions on the b-wave recorded by the ERG.

The b-wave amplitude is a very good indicator for the functional integrity of the retina, thus being a good indicator for biocompatibility of the tested irrigation solution. However, effects of the irrigation solutions on any specific site of the retina cannot be measured by the b-wave alone. To investigate the effects on the photorecep-

tor function alone, the a-wave amplitude using aspartate was examined.

During perfusion with BSS a significant decrease of a-wave amplitudes was noted. This effect was reversible at the end of the washout. Earlier studies from Moorhead et al. [20] on rabbit eyes support this data. Taking a closer look at the molecular composition of BSS one can see probable reasons for the decreased function, e.g. it has been shown that low or high potassium concentrations (10.1 mM KCl for BSS) significantly reduce the a-/b-wave amplitude [16]. Different buffering systems have a similar effect [17]. BSS has a potassium concentration of 10.1 mM, which alone could result in a reduction. This explains the reduction of the signal on the ERG. Looking at the long-term survival of the retinal ganglion cells BSS shows a negative effect compared to the control group (serum-

Fig. 4. Effects of the different irrigation solutions on the ganglion cells of isolated whole mounts. Retinas were stored at 4°C, protected from light for 24 h and stained using the Live/Dead viability/cytotoxicity kit. Increased number of fluorescent stained cells indicates more dead ganglion cells. Zeiss Aviovert 200. Magnification $\times 200$. Representative pictures.

free standard medium). These data are supported by studies from Araie and Kimura [21], who observed a negative effect on the barrier function of the retinal pigment epithelium and on the permeability of the retina. In contrast to this data, BSS is still widely used – mostly because of the lack of negative clinical experience. However, the data gathered in this study with an isolated bovine retina show that BSS is not an ideal irrigation solution.

During perfusion with PuriProtect no statistically significant changes in the a- or b-wave amplitudes were noted. This is supported by data from Lücke et al. [14] and from Sun and Xu [22], who could not show negative effects of taurine on the ERG and on cell cultures. Taking a look at the molecular composition of PuriProtect, which is very similar to BSS, this seems surprising. The potassium and calcium components with a concentration of 10.1 mM KCl and 3 mM CaCl_2 have different concentrations from those which have been proven best for maintaining physiological cell metabolism in previous studies [17, 18]. As mentioned before, a different concentration of potassium has been shown to have a negative impact on retinal function. In addition, a calcium concentration higher than 0.15 mM results in a decreased electrical signal, caused by exceeding the solubility product of hydroxyapatite $[\text{Ca}_5(\text{PO}_4)_3\text{OH}]$, a salt formed in phosphate-containing solutions. It has been shown that an increase in calcium concentration closer to physiological conditions results in a complete reduction of b-waves. This reduction was shown to be reversible by simply lowering the calcium condition again. The optimal concentration of 0.15 mM is empirically optimized [18]. Taking this into consideration, one would have expected a significantly decreased signal during and after the application of PuriProtect. It could be argued that a negative effect exerted by the molecular composition of PuriProtect is bolstered by the presence of taurine, which has been shown to be

Table 3. Effect of the irrigation solutions on the survival of retinal ganglion cells

Irrigation solution	Cell death ¹ , %	SD, %	p value
Control	69.6	3.9	–
Control with glutathione	36.3	13.9	0.004
BSS	84.0	12.4	0.06
PuriProtect	30.1	14.1	0.0014

¹ After storage of whole-mount preparations for 24 h at 4°C protected from light.

neuroprotective and to ameliorate the ERG signal [14, 21]. The protective effects of taurine on cell volume homeostasis and stress responses seem to be of particular importance [9, 10].

This assumption is supported by a significant decrease in cell death after 24 h at 4°C while protected from light. Storing the retina under these conditions does not correspond to the physiological situation, but low temperatures have been shown to decrease cell metabolism, prolonging cell survival. Therefore, data from these cell culture experiments should be regarded carefully. Our results match the data from Chen et al. [11], who have also reported a neuroprotective effect of taurine in cultured neurons. To take a closer look at the isolated function of taurine it was added solely to the standard solution. A significant increase in b-wave function, representing the inner neuronal network of the retina, was noted, agreeing with the results from previous perfusion studies [14]. These data are supported by the results of our cell culture experiments, by other studies showing positive effects on cell lines and by clinical experience. The mechanism by

which taurine acts has been discussed in the literature for many years without a clear answer. Our data support the assumption that taurine outbalances possible negative effects of PuriProtect caused by its molecular composition [22–24]. In general, the ERG data should be considered to be more valuable than the data from the cell culture experiments because it focuses on the functional analysis and is more reliable than histological, ultrastructural or biochemical analysis [25].

In conclusion, the use of a standard balanced salt solution as an intraocular irrigation solution is not recommended despite the positive clinical experience. It seems that a taurine-containing irrigation solution has, in gen-

eral, a more beneficial effect on the long-term survival of the whole-mount retina and thus may be a better option for vitreoretinal surgery.

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Disclosure Statement

The authors have no financial interests to disclose.

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