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In-vitro cytocompatibility and growth factor content of GBR/GTR membranes

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ABSTRACT

Objective. To assess the cytocompatibility of five commercially available xenogenic barrier membranes used for oral regenerative procedures and to determine the growth factor content of these membranes in-vitro.

Methods. Human mesenchymal stem cells (hMSCs) and immortalized periodontal ligament stem cells (PDL-hTERTs) were used to determine the cytocompatibility of xenogenic barrier membranes made of collagen (Biogide, BG, Geistlich Pharma AG, Switzerland; Biomend, BM, Zimmer Biomet, USA; Osseoguard OG, Zimmer Biomet, USA; OssixPlus, OX, Datum Dental, Israel) or extracellular matrix (ECM) (Dynamatrix, DM, Keystone Dental, USA) and of their eluates obtained by washing. Cells were cultured with previously washed and unwashed membranes (n=4) and in the medium used for washing (eluate). Cell proliferation at 3 days (eluates) and at 7 days (membranes) was assessed using the WST-1 cell proliferation kit. Growth factor content of the membranes was measured using multiplex ELISA.

Results. The eluate of BG and BM significantly inhibited proliferation of hMSCs, whereas DM and OX showed stimulating effects. The highest impact was observed for DM, its eluate doubled the cell proliferation of adherent cells when compared to the control ($p < 0.001$). The eluate of OG did not influence eluate cell cultures ($p > 0.05$). The presence of membranes had different impact on hMSCs and PDLs. hMSCs seem to be more resistant to the inhibitory effects of BG, OG and BM. hMSCs are only affected by OX, which actually stimulates hMSCs when the specimens are not washed previously. PDLs however proliferate significantly less once they are placed into culture with BM and OG as well as BG-not washed. Once BG is washed no inhibitory effect on PDLs was observed, however overall the washing of membrane samples prior to the placement into the cell culture did hardly have any effect on the outcome. The strongest inhibition of proliferation was shown with the BM and OG membrane in PDL-hTERTs ($p < 0.001$). Growth factor contents were quite similar quantitatively and qualitatively among the tested membranes with concentrations in the range of 50–500 pg/ml. Intriguingly DM contained considerably higher amounts of bFGF with up to 8000 pg/ml.

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Significance. The collagen membranes cross-linked with aldehydes show poor outcomes in PDLs while the collagen membrane cross-linked with polysaccharides generally shows promising results similar to the ECM-membrane DM in both membrane and eluate tests. The findings may be due to various factors, especially differences observed in composition, processing and bFGF content.

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1. Introduction

Guided bone regeneration (GBR) and guided tissue regeneration (GTR) are routine clinical procedures, but their successful application is highly dependent upon various factors, i.e. the experience of the clinician, the technique and the materials used as bone graft substitute or the barrier membrane [1–3]. Membranes are primarily used to prevent epithelial ingrowth into the hard tissue graft and to maintain the defect volume during osseous healing [4,5]. Currently available barrier membranes are commonly classified according either to their origin, i.e. synthetic or xenogenic, or their degradation dynamics, i.e. absorbable or non-absorbable. The latter commonly show complete absorption within several months largely mediated by enzymatic degradation [6,7]. In addition to synthetic materials with promising biocompatibility data as shown by Hoornaert et al., various xenogenic sources, i.e. collagen or extracellular matrix are used for the preparation of absorbable barrier membranes [8,9]. Native collagen membranes typically provide excellent biocompatibility but show inappropriate rapid degradation compromising the barrier function within periods of time shorter than needed for successful osseous regeneration. To extend the total degradation time and maintain the barrier function during the entire regenerative healing process collagen membranes are chemically modified, i.e. cross-linked [10]. Various substances are used to induce cross-linking within the collagen membranes among which are agents with considerable cytotoxicity, i.e. glutaraldehyde or formaldehyde [11–13]. Apart from cross-linking, also other components are expected to have a negative impact on cell proliferation, since also non-cross-linked membranes have been shown to have a certain inhibitory and anti-proliferative potential leading to the inhibition of cell migration [14]. The not cross-linked collagen membrane Bio-Gide (Geistlich Pharma, Switzerland) undergoes a purification and sterilization process, which according to the manufacturer include alkaline treatment at a pH > 13 for numerous hours as well as chemical processing steps including acids.

The aims of this study were to compare five xenogenic barrier membranes with respect to their cytocompatibility. The null-hypothesis I was that the specific type of the membrane does not have any impact on cell proliferation in-vitro, irrespective of the cell type used. The second null-hypothesis postulated that there is no difference between adherent or non-adherent cells. Further, it should be assessed whether the washing of these membranes causes changes of their biocompatibility. The null-hypothesis III was that washing does not affect cell proliferation in-vitro. Finally the growth factor content of the washing medium should be determined. The null

hypothesis IV postulated that no growth factors are detectable within the washing medium and that the medium itself has no effect on cell proliferation.

2. Materials and methods

2.1. Barrier membranes

Five commercially available xenogenic membranes were used for this study: Biogide (BG, Geistlich Pharma AG, Switzerland), Biomend (BM, Zimmer Biomet, USA), Dynamatrix (DM, Keystone Dental, USA), Osseoguard (OG, Zimmer Biomet, USA), OssixPlus (OX, Datum Dental, Israel). Detailed membrane characteristics are listed in Table 1. Specimens were prepared using a round-shaped punch of 13 mm diameter under sterile conditions.

2.2. Cell culture — membrane sample

Cell proliferation was tested separately with previously washed and unwashed membranes. For the proliferation measurements PDLs and hMSCs (12,000 cells/well, 700 μ l culture medium) were placed together with the membrane samples into 20 wells of four 24-well-plates each: PDL-washed, PDL-not washed, hMSC-washed, hMSC-not washed. Four cell culture samples without any membrane served as positive control. All samples were incubated for 7 days.

The tests were performed with human immortalized periodontal ligament progenitor cells (PDL-hTERT) [15] and human mesenchymal stem cells (hMSC, Lonza, LOT0000471980). The hMSCs donor was a black, 20-year old male.

Each type of membrane has been tested in culture in four replicates ($n = 4$) on both, hMSCs and PDL-hTERTs. In case of washed membranes the samples have first been washed in cell culture medium for 72 h, either in alpha-MEM (hMSC) or in DMEM (PDL-hTERT). For each type of membrane an aliquot of the washing medium (eluate) was frozen and stored at -80°C for the determination of signaling molecules. Again, for each type of membrane four previously washed samples have been placed into four ($n = 4$) cell culture samples of hMSCs and PDL-hTERTs. For the separate determination of the proliferation of cells adherent either to the membrane or to the bottom of the well each membrane sample has been transferred into new 24-well plates after 7 days. Afterwards the remaining culture medium has been carefully removed and replaced by 50 μ l WST-1 and 500 μ l fresh culture medium for each single well. Also the wells with the membrane samples received the same amount of WST-1 and culture medium. Following 1 h of additional incubation the specimens were finally discarded

Table 1 – Materials.

Membrane	Composition	Origin	Tissue	Cross-linked
BG	Collagen I	Porcine	Dermis	No
BM	Collagen I+III	Bovine	Deep flexor (Achilles) tendon	Gluteraldehyde
DM	Extracellular matrix	Porcine	Intestinal submucosa	No
OS	Collagen	Bovine	Deep flexor (Achilles) tendon	Formaldehyde
OX	Collagen	Porcine	Tendon	Sugar-based

Table 2 – 40 growth factors detected by the Quantibody® Human Growth Factor Array 1 Kit.

Each antibody is printed in quadruplicate horizontally								
1 2 3 4			1 2 3 4			1 2 3 4		
A	POS 1				POS 2			Amphiregulin
B	BDNF				bFGF			BMP-4
C	BMP-5				BMP-7			Beta-NGF
D	EGF				EGFR			EG-VEGF (PK1)
E	FGF-4				FGF-7 (KGF)			GDF-15
F	GDNF				GH			HB-EGF
G	HGF				IGFBP-1			IGFBP-2
H	IGFBP-3				IGFBP-4			IGFBP-6
I	IGF-1				Insulin			M-CSF R
J	NGFR (TNFRSF16)				NT-3			NT-4
K	Osteoprotegerin				PDGF-AA			PLGF
L	SCF				SCF R (CD117)			TGF alpha
M	TGF beta 1				TGF beta 3			VEGF-A
N	VEGFR2				VEGFR3			VEGF-D

and proliferation measurements were taken with a microplate reader (Tecan Infinite M200, Tecan Ltd, Switzerland). For statistical analyses the absorbances obtained from the well-adherent cells and the respective membrane were summed up to one cell proliferation absorbance value per membrane sample.

2.3. Cell culture — eluate samples

The biological effects of the washing medium (eluate without membrane) were tested on hMSCs only. An aliquot of 500 μ l of the eluate of each type of membrane was placed onto adherent hMSCs that have been previously cultured for 24 h or on non-adherent cells (12,000/well). After 96 h the eluate was removed, 50 μ l WST-1 and 500 μ l fresh culture medium added to each single well. Following 1 h of additional incubation the specimens were finally discarded and proliferation measurements were taken with a microplate reader as previously described (Tecan Infinite M200, Tecan Ltd., Switzerland). These experiments have been done for each type of membrane and cell culture stadium (i.e. adherent and non adherent cells) in four replicates ($n = 4$).

For quantification of signaling molecules in the barrier membranes a sandwich-based Human Growth Factor Multiplex ELISA Array (RayBiotech Life, GA, USA) able to detect 40 signal proteins has been used (Table 2). The set includes two slides with 16 sub-arrays each. 8 are needed for standards, so 8 samples per slide can be placed. Due to the similar composition of OG and BM and the results obtained in the cell proliferation assay, OG was not further included in this test. This allowed for two samples of each of the residual four mem-

branes (BG, BM, DM, OX) per slide, having total two slides (technical replicates) with two samples (biologic replicates) each per membrane. The Multiplex ELISA was run according to the instructions of use. A microarray scanner (Surescan DNA Microarray Scanner (G2505C), Agilent, USA) was used for measurements.

2.4. Statistical analysis

According to a priori analysis the power to detect a mean difference of 0.1 in WST-1 absorbance with a standard deviation (SD) of 0.05 at a confidence interval of 95% was 0.8 (One-way ANOVA (pairwise, 2-sided equality)). All data are given as mean \pm SD. Data sets have been tested for homogeneity of variances using Levene's test and for normal distribution using the Shapiro–Wilk test. For analysis of differences between groups One-Way ANOVA has been applied. Correction for multiple testing was done using the Bonferroni procedure. For all statistical procedures the level of significance was set at $p = 0.05$. Statistical analysis of data was performed with SPSS 24.

3. Results

3.1. Influence of barrier membranes on the proliferation of hMSCs and PDL-hTERTs

Membranes placed into cell culture for 7 days had different effects on hMSC and PDL growth. hMSCs growth is stimulated 2-fold by OX (0.458 ± 0.156 , Mean \pm SD) vs. Ctr-NW (0.239 ± 0.031 , Mean \pm SD) when not previously washed ($p < 0.001$). However all other membranes, washed or not washed, do not statistically significantly affect cell proliferation when compared to the respective control (Figs. 2 and 3). Nevertheless once washed and seeded with hMSCs (Fig. 3) there is a significant trend towards increased cell proliferation (Mean \pm SD) with DM (0.361 ± 0.075) and OX (0.3 ± 0.046) when compared to BM (0.154 ± 0.011), BG (0.165 ± 0.038), OG (0.181 ± 0.03) with DM showing statistically significantly higher proliferation than BM, BG, OG ($p < 0.05$).

PDL growth is more sensitive to membranes. As shown in Fig. 4, when not washed, BG, BM and OG are highly inhibitory ($p < 0.002$), DM and OX had almost no effect when compared to the control ($p = 1$). Once washed BM and OG still inhibit cell growth ($p < 0.007$), BG ($p = 1$), OX ($p = 0.647$) and DM ($p = 0.358$) do not differ statistically from control, despite a trend of DM towards cell growth stimulation (Fig. 5). In any case since membranes affect cell proliferation the null hypothesis I can be rejected.

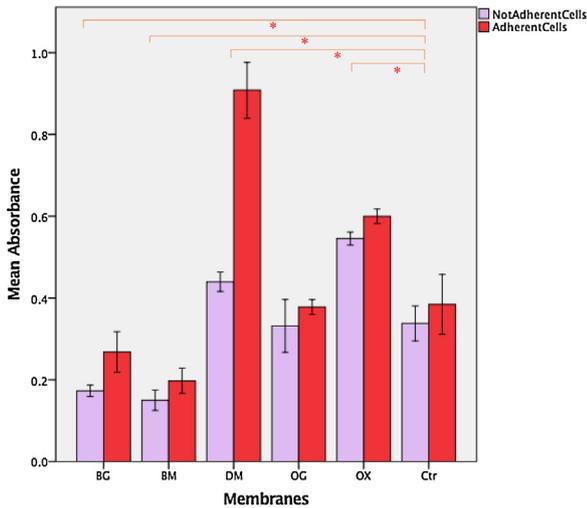


Fig. 1 – Mean absorbance when eluate was placed with not adherent cells in suspension or on previously attached hMSCs. * Indicate statistically significant differences when compared to control Ctr (p = 0.05). Error bars ±SD.

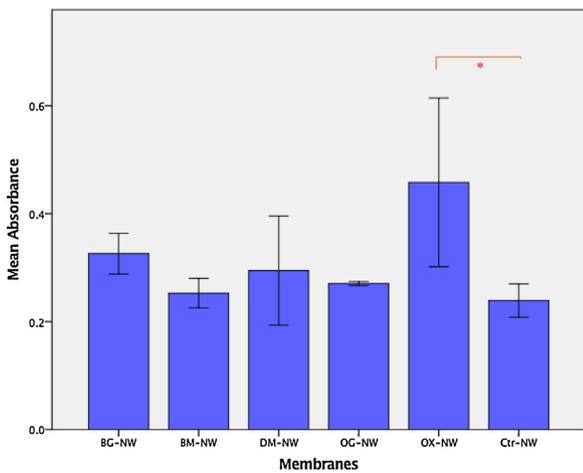


Fig. 2 – Mean hMSC cell proliferation with not washed membranes placed in culture. Statistical significant differences in comparison to the respective control group are marked with a * (p = 0.05). Error bars ±SD.

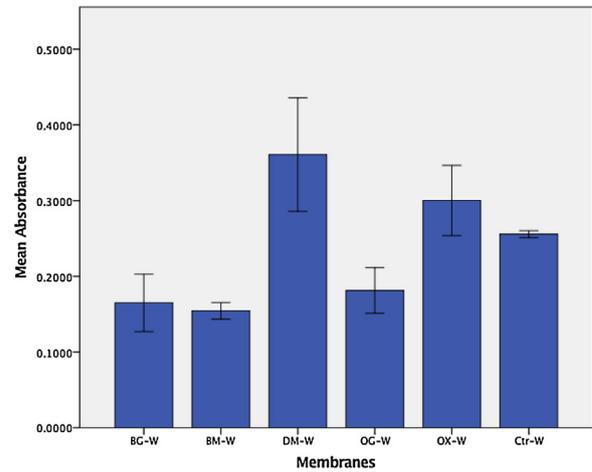


Fig. 3 – Mean hMSC cell proliferation with washed membranes placed in culture. No statistical significant differences in comparison to the respective control group (p = 1). Error bars ±SD.

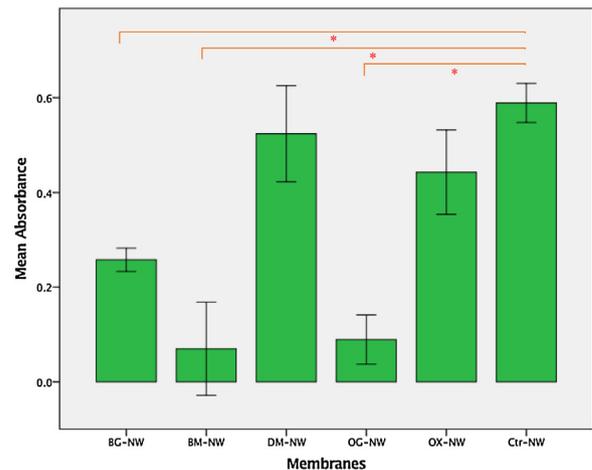


Fig. 4 – Mean PDL cell proliferation with not washed membranes placed in culture. Statistical significant differences in comparison to the respective control group are marked with a * (p = 0.05). Error bars ±SD.

3.2. Influence of membrane eluate on the proliferation of hMSCs and PDL-hTERTs

The effect of washing is limited, but there is some, so that hypothesis II can be rejected. To compare washed and not-washed data from different plates data was adjusted for the control proliferation rate. Relative cell proliferation was calculated by dividing the mean of each membrane by the mean control growth of the respective plate. hMSCs tend to grow better when membranes are not washed, whereas PDLs show higher proliferation values when membranes are previously washed. However statistical significant differences are rare and limited to two groups. DM results in statistically higher PDL growth, OX in less hMSC proliferation when DM and OX are washed and compared to the not washed equivalent group.

The membrane eluate does significantly effect proliferation of hMSCs, whether they are attached or not (Fig. 1). Nullhypothesis III is rejected. Both, the placement of the eluate to the samples of the adherent cells and to the non-attached cells influenced cell proliferation only in case of DM eluate. In addition there was observed a highly significant difference for DM when comparing adherent and non-adherent cells (p < 0.001). Thus, nullhypothesis IV can be rejected with respect to DM. While the OG eluate does result in similar cell growth as the control, BM and BG eluates inhibit, DM and OX stimulate cell growth statistically significantly (p < 0.05; Fig. 1). DM eluate when placed onto adherent hMSCs stimulates cell growth by factor 2.36 ± 0.18 (Mean ± SD), OX with both adherent and

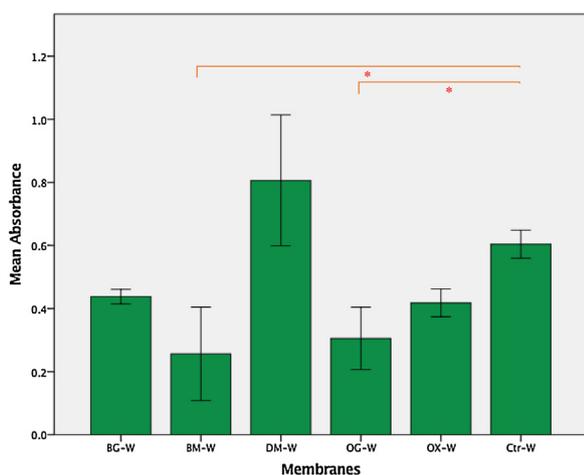


Fig. 5 – Mean PDL cell proliferation with washed membranes placed in culture. Statistical significant differences in comparison to the respective control group are marked with a * ($p = 0.05$). Error bars \pm SD.

non-adherent application by factor 1.56 ± 0.05 and 1.62 ± 0.05 (Mean \pm SD) respectively. BG and BM eluates reach 44–70% of the control cell growth.

3.3. Determination of signaling molecules in barrier membranes

With respect to growth factors hypothesis IV can be rejected. As a proof-of-principle the Multiplex Elisa revealed that both collagen and extracellular membranes contain some sort of growth factors, however in relatively low concentrations in the range of 50–500 pg/ml with high standard deviation (SD). Box plots are shown in Appendix A. The most common growth factors are IGFs, FGFs and BMPs. DM, the only extracellular matrix membrane, contains relatively high concentrations of bFGF (5000–8000 pg/ml).

4. Discussion

This study shows a significant impact of several barrier membranes on cell proliferation and – within the limitations of this study – that membranes, despite processing and sterilization, still contain considerable amounts of various growth factors.

The effect on cell proliferation depends on the membrane but also on the cell type. Anyhow the results are in line with observations in previous studies. Takata et al. showed that membranes do have an impact on cell behavior and may influence bone regeneration [14]. That may be the reason that implants placed into augmented bone show more crestal bone loss than implants placed into pristine bone, underlining the importance of optimizing materials used for regenerative procedures [16].

In addition, this study reveals some interesting new findings due to the number and type of membranes included. A strength of this study is that the membranes tested have a different composition and some are cross-linked by various techniques. Taken together the data show consistently that OX

and DM tend to enhance proliferation. On the contrary, for BG, BM and OG the proliferation was unaffected or even impaired in presence of a membrane. Since BM and OG are cross-linked xenogenic collagen membranes the negative effect on cell proliferation might have been caused by remnants of the agent used for cross linking. Formaldehyde and glutaraldehyde have been shown to be considerably cytotoxic and to impede cell proliferation [6,12,17,18]. However, also OX refers to the group of cross linked collagen membranes, but cross-linking of OX is based on polysaccharides. Probably these polysaccharides are less interfering with the viability and proliferation of cells in-vitro. Rothamel et al. showed better results for OX and BG when compared to BM, however they all resulted in highly significantly lower cell counts for PDL fibroblast as well as osteoblast-like cells than the control [12].

In another study BG, which is not cross-linked and hence expected to have good cytocompatibility, showed some negative effect on cell proliferation and cell migration [14]. So far the specific reasons why BG exerts negative effects on cell viability remain ambiguous, one possible reason may be the porcine origin of the BG membrane as discussed by Moura et al., who observed lower monocyte proliferation for BG when compared to control [19].

Previous studies are partially contradictory when it comes to BG cytotoxicity. Liu et al. reported good biocompatibility for BG using hMSC cultures as experimental model [20]. Willershausen et al. showed a similar positive effect on cell proliferation by BG in-vitro [21]. Standardized models with a variety of cell types are needed to clarify why BG in some circumstances would act cytotoxic or not.

In any case the release of potentially cytotoxic substances used for cross-linking of BM and OG cannot sufficiently explain the poorer biological compatibility of these membranes, since the eluate of OG did not affect the cell proliferation. On the other side washing of OG for 72 h may just not be enough to dissolve the cytotoxic components.

Herein DM and to a lesser extent also OX seemed to stimulate cell proliferation considering both, hMSCs and PDL-hTERTs. DM is derived from the extracellular matrix of small intestinal submucosa (SIS) and seems to contain relatively high amounts of bFGF according to the results of the multiplex ELISA. bFGF may have played a role in this study resulting in relatively good results when compared to control, possibly also due to stimulation of the secretion of other endogenous proteins such as VEGF. In the membrane supernatant itself VEGF or VEGFR were not detected at all. Nevertheless previous studies have actually shown that bFGF released from sterilized small intestinal submucosa-matrix maintains its biological activity leading to an increase in the VEGF-secretion by mouse fibroblasts [22]. Increased VEGF secretion may lead to an improved vascularization during healing as observed by Liu et al. Two kind of matrices (SIS and dermal matrix) were seeded with adipose-derived stem cells and observed (i) an increased secretion of VEGF and (ii) increased vascularization capacities in a mouse model [23].

OX shows promising results in-vitro behaving similar to ECM despite the lack of bFGF, possibly due to the cross-linking mechanism and some other residual growth factors, which however do not seem to differ from the other collagen mem-

branes tested both quantitatively and qualitatively. To allow for proper analyses and conclusions the companies should give detailed insight into the membrane composition.

Further tests with higher sample sizes are needed to clarify the content of this ECM-membrane and the collagen membranes and to determine if the healing and bone regeneration are actually influenced by the membrane. Clinically the membrane may determine whether complete regeneration is obtained and whether the volumetric dimension of the

graft remains stable over time. Especially in GTR cases proper regeneration at the most coronal portion of the grafted defect right at the membrane is essential to prevent re-pocketing at the grafted site long-term.

Appendix 1.

See Fig. A1.

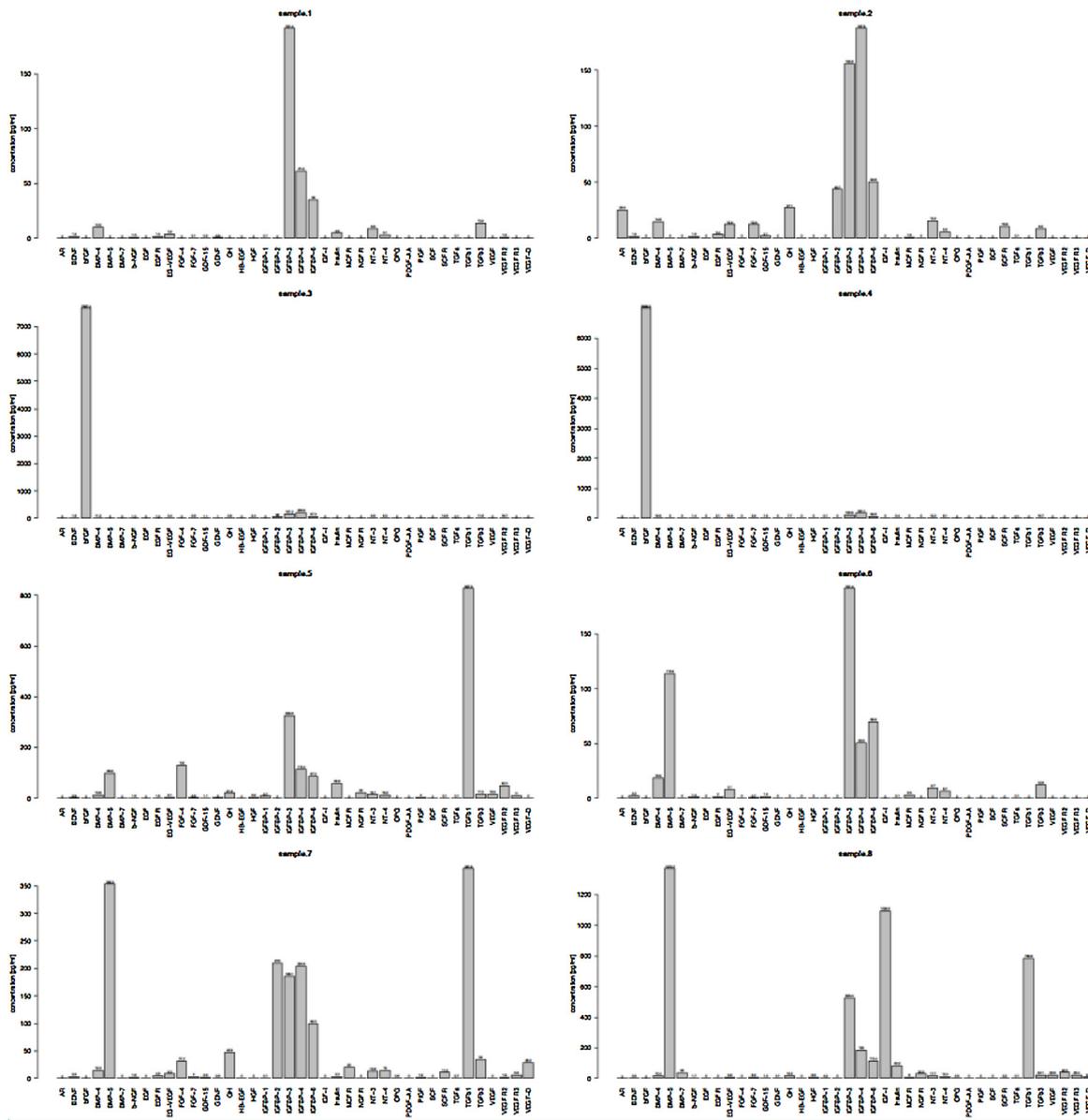


Fig. A1 – Concentrations of 40 growth factors in 4 membranes (n = 2). Sample 1, 2 = OX, sample 3, 4 = DM, sample 5, 6 = BM, sample 6, 7 = BG.

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