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ORIGINAL RESEARCH

In vitro Effects of Gadoteric Acid and Iobitridol Toxicity and Platelet-Rich Plasma-Mediated Protection in Human Annulus Fibrosus and Nucleus Pulposus Cells

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Study Design: Laboratory study.

Objective: Intervertebral disc (IVD) degeneration, a major cause of chronic low back pain characterized by reduced viability of Annulus Fibrosus (AF) and Nucleus Pulposus (AP) cells, can be treated with platelet-rich plasma (PRP), although confirming needle placement with radiopaque contrast, commonly used for this purpose, may negatively affect cellular viability. The objective of this study was to evaluate the cytotoxic effects of gadoteric acid (GA) and iobitridol (IB), commonly used contrast agents, and the protective potential of two PRP formulations: standard PRP (sPRP) and balanced protein-concentrate plasma (BPCP).

Methods: AF and NP cells were exposed to varying concentrations of GA and IB (0–100% v/v) for 1 and 2 hours, with or without 10% (v/v) PRP supplementation. PRP from five donors was pooled (n=1), and experiments were performed in technical triplicates (n=3). Cell viability was measured by luminescence-based RealTimeGlo MT assay. The difference among the groups was analysed and multivariable regression was performed.

Results: Both contrast agents appeared to exert dose- and time-dependent cytotoxicity. PRP was associated with improved cell viability, with BPCP appearing to provide increased viability under exposure to higher contrast concentrations. Multivariate regression analyses indicated that contrast concentration, exposure time, and PRP type were key determinants of cell viability.

Conclusion: These findings appear to underline the importance of minimizing contrast agent exposure during IVD procedures and suggest that PRP formulations, particularly BPCP, may provide improved cell viability against contrast-induced cytotoxicity. Future in vitro and in vivo studies will be needed to further validate these results and assess their clinical applicability.

Keywords: platelet-rich plasma, iobitridol, gadolinium, intervertebral disc, annulus fibrosus, nucleus pulposus, cell viability

Introduction

The intervertebral disc (IVD) is a key structure in the spine, facilitating mobility and providing cushioning between vertebrae. It is composed of two main regions: the annulus fibrosus (AF), a dense, fibrous outer ring, and the nucleus pulposus (NP), a gelatinous core that acts as a shock absorber.[1] Degeneration of the IVD, often due to mechanical stress, age, or injury, leads to a loss of structural integrity and is a major cause of chronic low back pain, which affects a significant portion of the global population.[2] Although various treatments exist, including surgery and conservative management, these therapies often do not fully restore IVD function or alleviate pain in the long term.[3] The cellular viability of both the human annulus fibrosus (hAF) and human nucleus pulposus (hNP) is critical for IVD health. The

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hNP, composed mainly of notochordal and chondrocyte-like cells,[4] plays a crucial role in maintaining the IVD's ability to absorb compressive forces.[5] On the other hand, the hAF, which consists of fibroblasts and collagen fibers, provides structural stability and resists radial and axial stress.[5] Both regions are highly susceptible to degeneration due to decreased cell activity and matrix production, leading to decreased disc height, hydration loss, and, ultimately, pain.[6]

Intradiscal treatments, aimed at addressing degenerative processes and disc herniations, often rely on the use of contrast agents to assess needle placement. Diagnostic discography is sometimes used prior to or during treatment, involving the injection of contrast material into the NP to analyze the integrity IVD and identify pain-generating discs. Intradiscal drugs used in discography procedures may contribute to disc degeneration, beyond the physical damage associated with the puncture.[7] The use of contrast agents, such as gadoteric acid-based (GA) and Iobitridol (IB), allows the visualization of tissues in imaging studies, facilitating interventions. The former is widely used in magnetic resonance imaging (MRI) to enhance visualization of different tissues, including the musculoskeletal system.[8] IB, a non-ionic iodinated contrast, has also been used for imaging purposes, with its application in the spine providing valuable diagnostic.[9] These agents allow for detailed imaging of disc degeneration, with particular focus on the hydration status and vascularity of the IVD.[10] GA and IB have been shown to be safe for general use, with minimal adverse effects reported in clinical studies.[11,12]

Nowadays, several pharmaceutical formulations containing GA and IB have been commercialized as contrast agents for medical imaging. Clariscan® is a gadolinium-based contrast agent containing GA. The mean dose of Clariscan is 0.1 mmol/Kg and 0.2 mL/Kg.[13,14] Xenetix® is an iodinated, non-ionic, low-osmolar contrast agent containing IB. The dose of IB is determined according to the patient's body weigh.[9,15,16] Both contrasts are eliminated by urinary excretion. However, due to the limited research on their direct impact on IVD tissue, caution is advised when considering their use in procedures involving this area. Further studies are needed to elucidate any potential risks associated with their application in IVD imaging or interventions. While its effects on IVD tissues remain largely unexplored, studies have indicated that iodinated contrast agents like IB has been shown to be toxic to renal tissue[17,18] and recent research suggests that GA may have direct effects on tissue healing.[19]

Platelet-Rich Plasma (PRP) is an autologous blood derivative concentrated with platelets, which has shown promise in promoting tissue healing and regeneration. PRP is rich in bioactive molecules such as growth factors (GFs) that depends on platelets, including platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β) and vascular endothelial growth factor (VEGF) and GFs that are only present outside the platelets like insulin-like growth factor (IGF-1), except for the hepatocyte growth factor (HGF) which is present outside and inside platelets. Those GFs are involved in tissue repair and anti-inflammatory responses. In addition, new generation PRP products have emerged as a result of research and development over the last few years, such as the Balanced-Protein Concentrate Plasma (BPCP). Unlike standard PRP (sPRP) formulations BPCP concentrated not only platelet molecules but also extraplatelet ones, demonstrating higher cell proliferation capacity and improving the anti-inflammatory profile.[20] The main difference between them is that sPRP contains a two-fold of intraplatelet molecules while the BPCP contains a two-fold of both, extraplatelet and intraplatelet molecules.

In the context of the IVD, PRP has been investigated for its potential to improve cellular viability in both the hAF and hNP by stimulating extracellular matrix (ECM) synthesis and reducing inflammation.[21,22] Studies have demonstrated that PRP can enhance the proliferation and differentiation of disc cells, such as annulus fibrosus cells and nucleus pulposus cells, both of which play a critical role in maintaining disc integrity.[23,24] In vitro studies have shown that PRP application results in increased collagen production and improved cell viability within the IVD, potentially reversing degenerative changes.[25] Furthermore, animal models have suggested that PRP injection into degenerated IVDs can reduce pain and promote tissue regeneration.[25,26] It is well known that PRP has shown potential protective effects against the toxicity induced by local anesthetics and corticosteroids in IVD and tenocytes, too.[27–32] Therefore, the use of PRP in combination with contrast agents could offer protective capacity to the tissue degeneration generated by contrasts.

Therefore, it is proposed to investigate the protective effect of of two different PRP formulations (sPRP and BPCP) together with contrast agents to reduce its toxicity effect. Both PRP formulations have a moderate platelet concentration (x2) without leukocytes. In addition, BPCP contains twice the amount of extraplatelet molecules. For that, AF and NP cells were exposed to varying concentrations of GA and IB (0–100% v/v) for 1 and 2 hours, with and without 10% (v/v)

PRP supplementation. Cell viability was measured by luminescence-based RealTimeGlo MT assay and multivariable regression. We hypothesize that contrast agents have a dose and time-dependent toxicity in hAF and hNP cells in the IVD. Moreover, combining contrast agents with sPRP or BPCP cell viability can be enhanced, specially using BPCP.

Materials and Methods

Donors

Five healthy donors were selected ranging in age from 24 to 55 years-old. Whole blood was withdrawn into tubes of 9 mL containing 3.8% (w/v) sodium citrate. With the aim of reduce the inter-patient variability and minimize assay complexity, a single plasma pool was made by mixing all the patients' plasma in the same proportion. This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of the OSI Araba (2024–023, 30 May 2024). Informed consent was obtained from all subjects involved in this study.

Standard Platelet-Rich Plasma Preparation

Obtention of the sPRP was done by using the commercially available platelet-rich in growth factor kit (PRGF-Endoret®, Vitoria-Gasteiz, Spain) by centrifuging 9 mL of blood at 580 x g for 8 min at room temperature (RT). Then, collection of 2 mL of plasma fraction present over the red blood cell fraction was done, avoiding collecting white blood cells from the "buffy coat" layer. Finally, platelet-containing plasma pool was prepared mixing all obtained suspensions from all donors in the same proportion.

To ensure that the process is being carried out correctly, it is necessary to measure platelet levels in the pool. For the analysis of the platelet content a Mindray BC-20s haematology analyser (Mindray, Shenzhen, China) was used. Finally, 10% of $CaCl_2$ (20 μL mL^{-1}) was added to sPRP-pool for the release of platelet content and clot formation. The resulting platelet releasate was filtered with a Minisart ® NML Plus 0.2 μm filter (Sartorius, Goettingen, Germany) to sterilize it.

Balanced Protein-Concentrate Plasma Preparation (BPCP)

As described by Mercader et al[33] the BPCP was prepared by centrifuging 9 mL of whole blood at 1200 × g for 8 minutes at RT. The entire plasma fraction was carefully collected while avoiding the inclusion of red and white blood cells. The concentration parameters were designed to produce a basal plasma fraction with platelet and extraplatelet levels comparable to those in whole blood, ensuring that the final concentration of the BPCP would double the baseline levels. Subsequently, a platelet-containing plasma pool was prepared by combining all obtained suspensions from all donors in equal proportions, under the assumption that platelet levels equal those of blood basal levels. The resulting plasma pool was then exposed to 0.125 g mL^{-1} of hydroxyethyl acrylamide (HEAA) hydrogel, allowing water absorption to occur for 5 minutes. To remove the hydrated hydrogel particles, the mixture was filtered through a 100 μm filtration unit placed over a 50 mL Falcon tube. The tube was then centrifuged at 500 × g for 2 minutes at RT to separate the platelet-containing plasma pool from the hydrogel particles. The final product obtained is referred to as BPCP.

To ensure that the process is being carried out correctly, it is necessary to measure platelet levels in platelet-containing plasma pool before and after being put in contact with HEAA hydrogel. For the analysis of the platelet content a Mindray BC-20s haematology analyser (Mindray, Shenzhen, China) was used. Finally, 10% of $CaCl_2$ (20 μL mL^{-1}) was added to the BPCP formulation for the release of platelet content and clot formation. The resulting platelet releasate was filtered with a Minisart ® NML Plus 0.2 μm filter (Sartorius, Goettingen, Germany) to sterilize it.

Human Annulus Fibrosus (hAF) Cell Cultures

hAF cells (Innoprot, Derio, Bizkaia) were kept in the incubator at 37 °C and 5% CO_2 atmosphere. Cells were grown in Nucleus Pulposus Cell Medium (NPCM, Innoprot, Derio, Bizkaia) supplemented with Nucleus Pulposus Cell Growth Supplement (NPCGS) and penicillin/streptomycin (P/S) at 1% (v/v) each, and 2% of fetal bovine serum (FBS) as recommended by the manufacturer. All experiments involving hAF were performed between passage 5–7.

Human Nucleus Pulposus (hNP) Culture

hNP cells (Innoprot, Derio, Bizkaia) were kept in the incubator at 37 °C and 5% CO_2 atmosphere. Cells were grown in NPCM (Innoprot, Derio, Bizkaia) supplemented with Nucleus Pulposus Cell Growth Supplement (NPCGS) and penicillin/streotinycin (P/S) at 1% (v/v) each, and 2% of fetal bovine serum (FBS) as recommended by the manufacturer. All experiments involving hAP were performed between passage 5–7.

Cell Viability Assay

Cellular toxicity of both GA (Clariscan™, GE Healthcare Bi-Sciences, Madrid, España) and IB (Xenetix®, Guerbert, Cedex, France) was evaluated in hAF and hNP under sterile condition. 5,000 cells were seeded per well in a 96-well cell culture plate (655083; Greiner, Kremsmünster, Austria) with different contrast medium percentages (20, 40, 60, 80 and 100%, Figure 1) for 1 and 2 h. Contrast dilutions were prepared in NPCM basal medium with 1% (v/v) NPCGS and 1% (v/v) P/S without FBS. Dilutions were supplemented with plasma (10% v/v sPRP or BPCP) or without plasma (serum free, SF). Washes were performed before adding the treatment.

Cellular viability was assessed in triplicate using the RealTime-Glo MT Cell Viability Assay (Promega, Fitchburg, USA). This assay relies on the reducing capacity of metabolically active cells, which convert a synthetic substrate into a luminescent product. Luminescence measurements were obtained using a TECAN Infinite 200 PRO plate reader (TECAN, Zurich, Switzerland). The luminescence intensity is directly proportional to the number of viable cells present in the assay.[34]

Statistical Analysis

The main variable analyzed was cell viability, for which a convenience sample was used. Distribution of the samples was assessed by Shapiro–Wilk's normality test. The different variables were determined by the mean and the standard deviation for parametric data. The difference among the groups was analysed *t*-test or ANOVA followed by Tukey/Dunnett's multiple comparison test. Nevertheless, for non-parametric data, Kruskal Wallis test was performed. Data were considered statistically significant when $p < 0.05$. Finally, to evaluate the weight of each variable on cell viability, a multivariate regression was performed. For each condition, three technical replicates were measured from a single pooled biological sample derived from five donors. These replicates were not biologically independent, but were included in this analysis to explore trends and relationships between predictors and the response variable. Linear regression models were fitted including the main predictors without interactions. Multicollinearity was assessed via Variance Inflation Factors (VIF). For multiple comparisons, p-values for the four predictors were adjusted using Bonferroni correction ($p \times 4$), and confidence intervals were correspondingly adjusted (98.8% CI; $p < 0.0125$). Results were considered exploratory and descriptive due to the use of a single pooled sample. GraphPad Prism® software 9.5 version (San Diego, CA) was used for the statistical analysis.

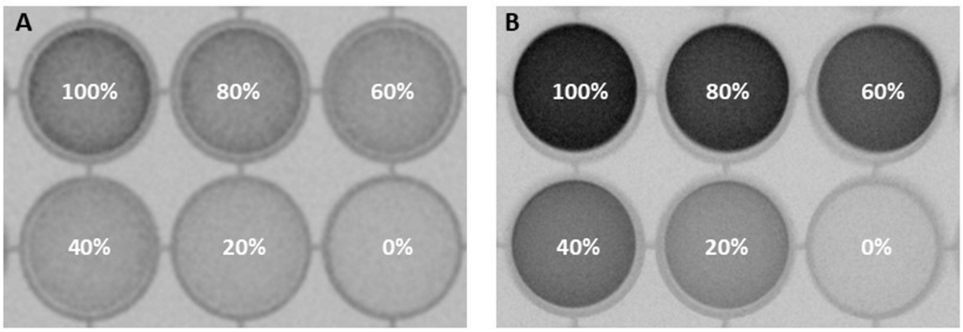

Figure 1 X-ray images showing varying concentrations of both, GA and IB contrast mediums. Different concentrations in a range of 0–100% of GA (**A**) and IB (**B**) were measured by X-ray with Ziehm Vision RFD 3D (Ziehm Imaging, Nürnberg, Alemania) equipment. Dilutions were made in NPCM.

Results

sPRP and BPCP Platelet Level Concentration

Platelet levels were measured in blood-derived plasma fraction pool, sPRP pool and BPCP pool. Platelet analysis showed that platelet content in both PRPs was twice as high comparing to plasma levels, being this similar to blood basal levels (120×10^3 platelets μL^{-1}). Platelet concentration of sPRP was 234×10^3 platelets μL^{-1} and for BPCP 224×10^3 platelets μL^{-1}. Neither of them had leukocytes or erythrocytes. According to the UCS (Universal Coding System) for PRP studies described by Kon et al[35] the products used in this study were 12-00-11 (Table 1).

Effect of GA and IB on the Cell Viability of hAF

In order to analyze the cytotoxic effect of GA and IB, hAF cells were exposed to different concentrations of contrast for 1 and 2h. Both, GA and IB showed cellular toxicity at all concentrations used, compared to unexposed cells ($p < 0.05$) (Figure 2). Regarding to cells exposed for 1h, higher concentrations of GA showed higher cell toxicity (Figure 2A). In contrast, cell viability was more stable in hAF cells exposed to IB for 1h (Figure 2B). Nevertheless, when comparing both contrasts, significant differences were found at higher concentrations (60, 80 and 100%) ($p < 0.05$) (Figure 2C).

Table 1 Summary of Characteristics for PRP

	sPRP	BPCP
1. **PRP Preparation**		
Initial blood volume	9 mL per tube	9 mL per tube
Anticoagulant	Sodium citrate 3.8% (wt/V)	Sodium citrate 3.8% (wt/V)
System	Close	Open
Centrifugation	Yes	Yes
Number	1	1
Speed	580 x g – 8 min	1200 x g – 8 min
Water absorption	No	Yes
Method		HEAA hydrogel
Hydrogel concentration		0.125 g mL^{-1}
Contact time		5 min
Final PRP volume	2 mL per tube	2 mL per subject
2. **PRP Characteristics**		
PRP Type	12-00-11	12-00-11
Platelets	234 x 10^3 μL^{-1}	224 x 10^3 μL^{-1}
Red Blood Cells	<0.01 x 10^6 μL^{-1}	<0.01 x 10^6 μL^{-1}
White Blood Cells	<0.05 x 10^6 μL^{-1}	<0.05 x 10^6 μL^{-1}
Neutrophils	–	–
Lymphocytes	–	–
Monocytes	–	–
Eosinophils	–	–
Basophils	–	–
Activation	$CaCl_2$ (10% wt/vol)	$CaCl_2$ (10% wt/vol)
3. **Application Characteristics**		
Dose	10%	10%
Direct/Indirect	Direct	Direct
Cell line	hAF and hNP	hAF and hNP

Notes: The product added to the cell cultures was the platelet releasate obtained after activation of PRP with calcium chloride (10%). BPCP doubled the standard PRP in the levels of extra-platelet molecules previously described by Sanchez et al (REF 20 of the manuscript).

Abbreviations: sPRP, Standard Platelet-Rich Plasma; BPCP, Balanced Protein-Concentrate Plasma; HEAA, hydroxyethyl acrylamide; hAF, Human Annulus Fibrosus; hNP, Human Nucleus Pulposus

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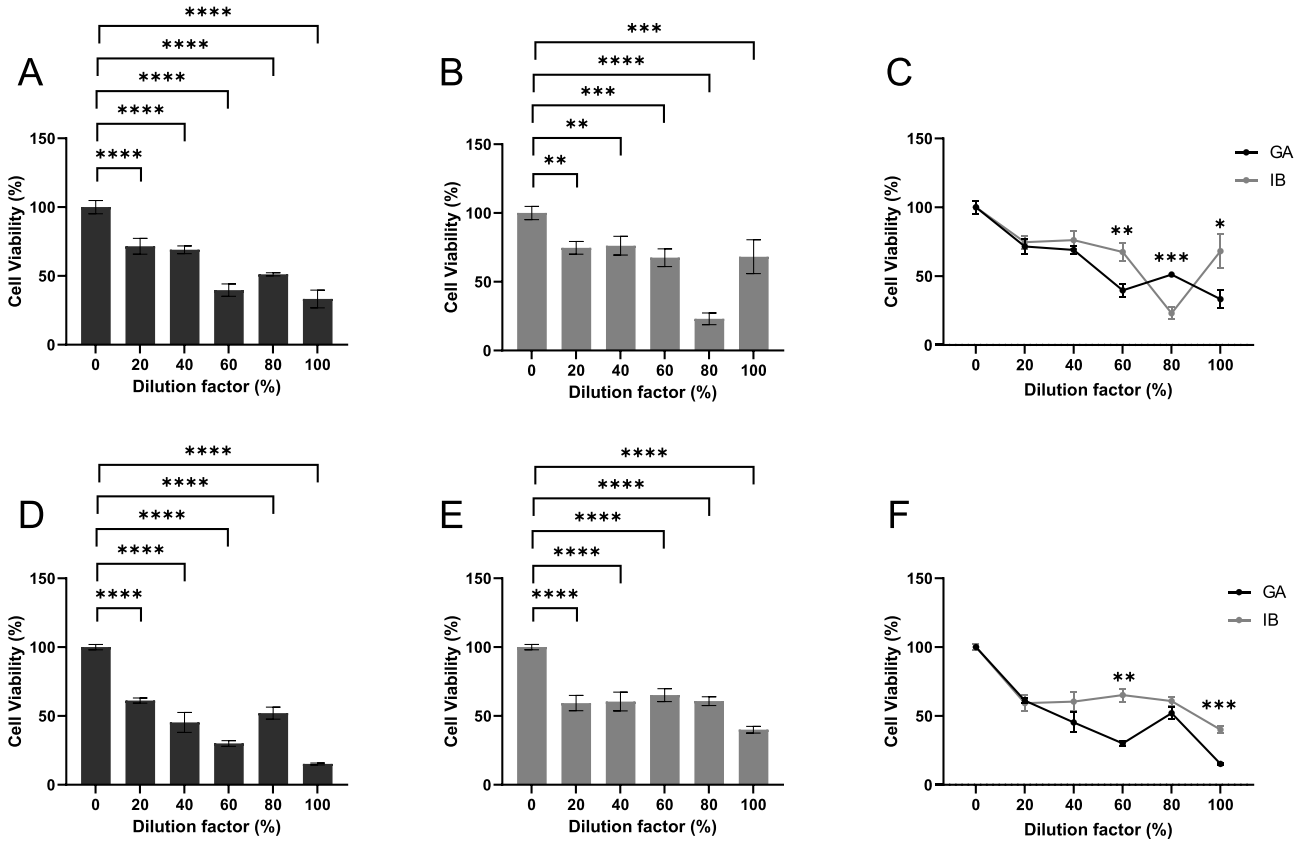

Figure 2 Effect of different concentrations of gadoteric acid (GA) and iobitridol (IB) on hAF cell viability. Measurement of cell viability (%) on cells exposed to GA (**A**) IB (**B**) and the comparison of the effect of both of them (**C**) for 1h. Same analysis was carried out at 2h (**D**–**F**). Each measurement was performed in triplicate. Cell culture medium was used as a negative control. Error bars = standard deviation (n = 3). Statistically significant differences were calculated using one-way ANOVA test (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

With respect to cells exposed for 2h, the same pattern achieved in the 1h condition was observed in the two contrasts (Figures 2D and E). When comparing both GA and IB at 2h, significant differences were observed in cells treated with 60% and 100% contrast ($p < 0.05$) (Figure 2F).

Effect of GA and IB Together with sPRP and BPCP on the Cell Viability of hAF

With the aim of studying the effect of the interaction between contrasts and PRP, different concentrations of GA and IB were made with cell culture medium supplemented with 10% of sPRP or BPCP. hAF cells were exposed to both 1h and 2h. The viability of cells in contact with the contrasts improved when cultured with sPRP and BPCP ($p < 0.05$). In all conditions, sPRP increased significantly cell viability at lower concentrations of both GA and IB. Nevertheless, improvement of cell viability was inversely proportional to contrast concentration. Regarding BPCP, a lower but more stable cell viability enhancement capacity was observed at both low and high contrast concentrations (Figure 3A–D).

Effect of GA and IB on the Cell Viability of hNP

GA and IB exhibited cellular toxicity across all tested concentrations in comparison to unexposed hNP cells ($p < 0.05$). Moreover, higher concentrations of both contrasts demonstrated increased cellular toxicity in 1h condition (Figures 4A and B). Significant differences were found between two contrasts in 20% and 60% conditions ($p < 0.05$) (Figure 4C). Regarding to cells exposed for 2h, the same pattern observed under the 1h- condition was described both GA and IB exposed cells ($p<0.05$) (Figures 4D and 4E). Upon comparison of GA and IB, significant differences were observed in cells treated with 40, 60, 80, and 100% contrast ($p<0.05$) (Figure 4F).

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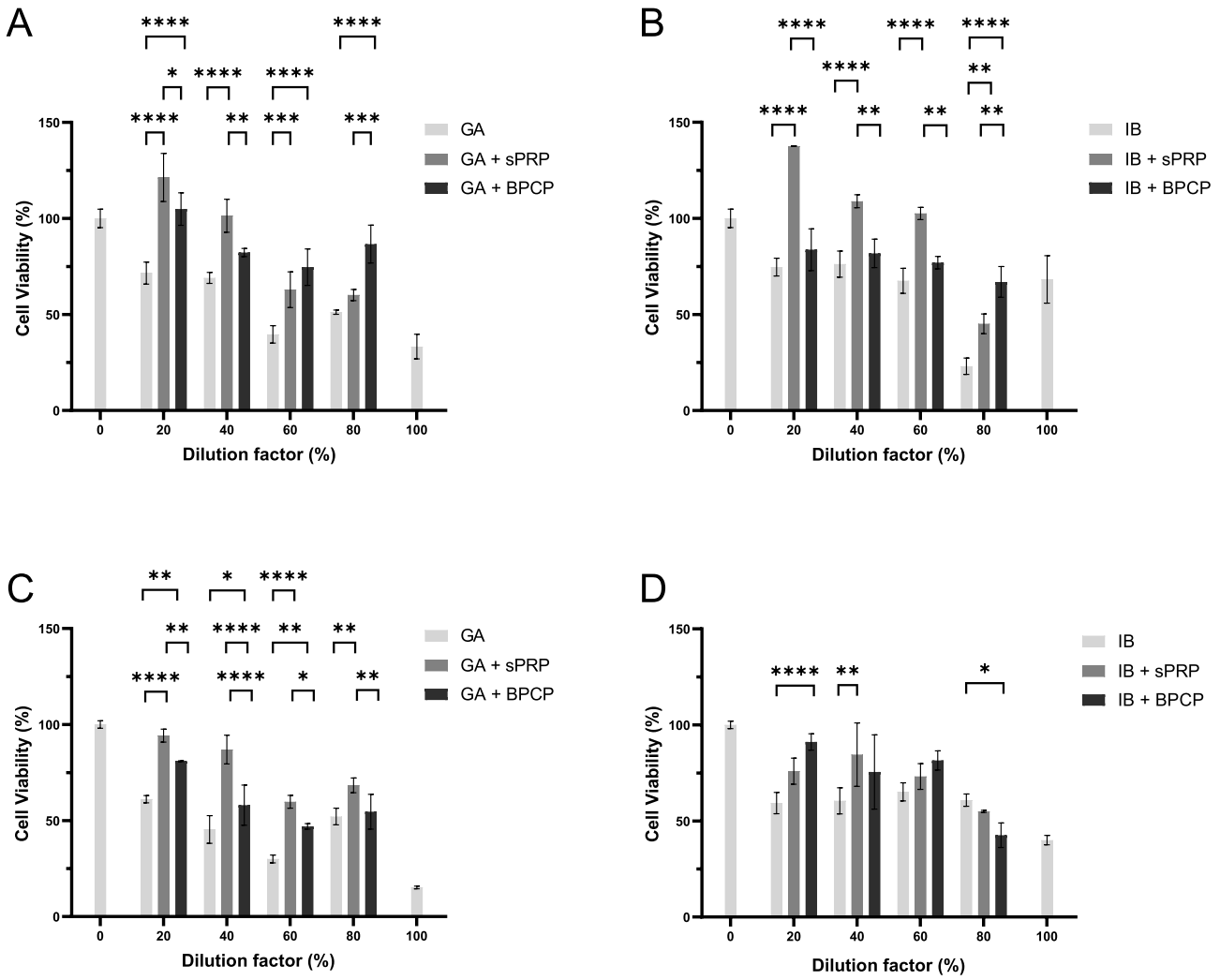

Figure 3 Study of interaction- effect of gadoteric acid (GA) and iobitridol (IB) with sPRP or BPCP on hAF cell viability. Measurement of cell viability of hAF exposed to different concentrations of GA (**A**) and IB (**B**) combined with 10% sPRP or BPCP supplemented cell medium for 1h. Same analysis was carried out for 2h (**C** and **D**). Cell culture medium was used as a negative control. Each measurement was performed in triplicate. Error bars = standard deviation (n = 1). Statistically significant differences were calculated using one-way ANOVA test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Effect of GA and IB Together with sPRP and BPCP on the Cell Viability of hNP

The effect of the interaction between both contrasts and sPRP and BPCP on the hNP cell viability was analyzed. In all conditions, both sPRP and BPCP increased significantly cell viability at lower concentrations of both GA and IB ($p<0.05$). Moreover, both sPRP and BPCP improved hNP cell viability similarly (Figure 5A–D).

Relationship of the Variables Influencing Cell Viability

With the main objective of finding out the influence of the variables as a whole (contrast concentration, exposure time, cell line and use or not of PRP) affect cell viability, a multivariate regression was performed (Table 2).

Analysis showed that the high concentration of contrasts, high contact time and not using PRP negatively affected cell viability. However, the line is not so important as both hAF and hNP show similar cell viability.

Based on the knowledge that the use of PRP and the amount of contrast has a significant influence, another multivariate regression (Table 3) was performed to analyze cell viability under various concentrations of contrast media (20%, 40%, 60%, 80%) and the use of both types of contrasts (GA and IB) and PRPs (sPRP and BPCP).

This multivariate regression analysis revealed again that exposure time had a significant negative impact on cell viability at all contrast concentrations, with prolonged exposure (2h) consistently reducing viability, particularly at higher

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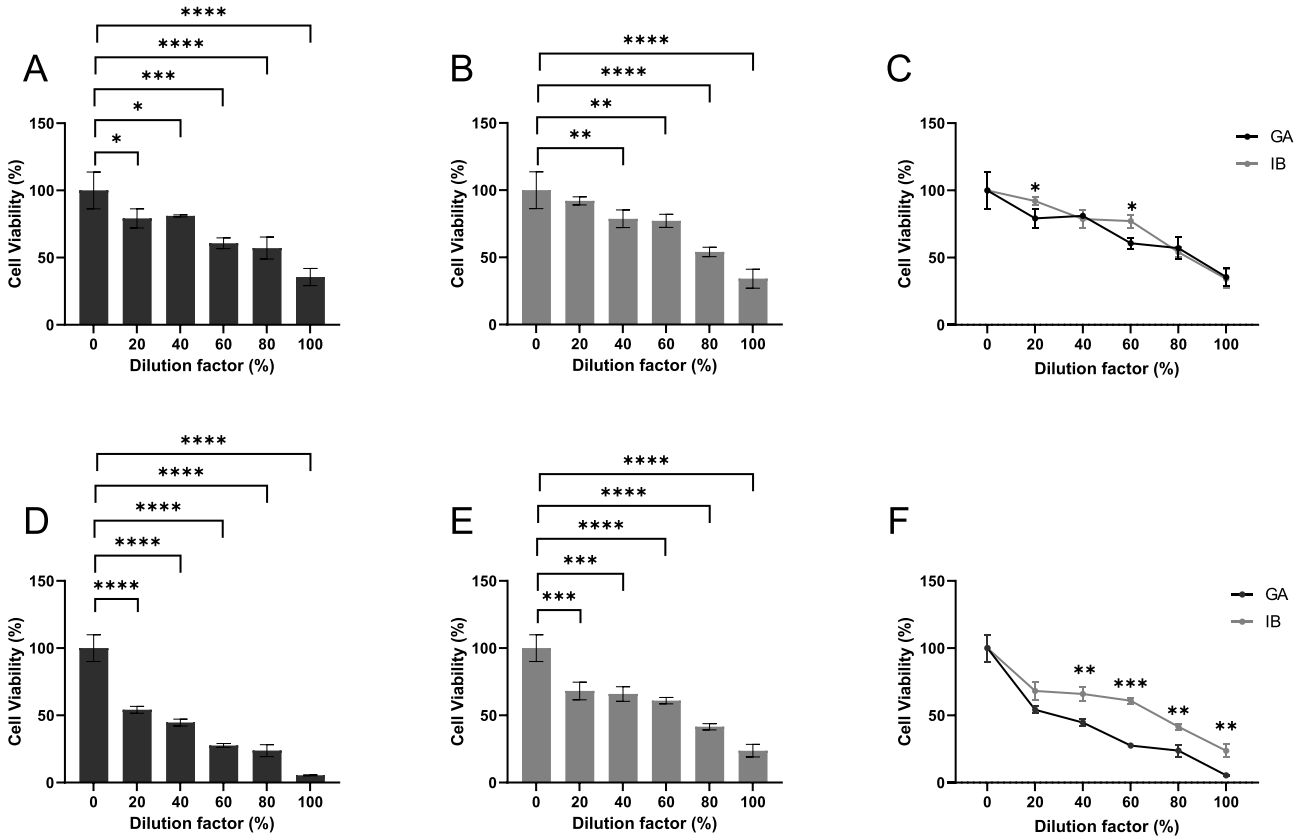

Figure 4 Effect of different concentrations of gadoteric acid (GA) and iobitridol (IB) on hNP cell viability. Measurement of cell viability on cells exposed to GA (**A**), IB (**B**) and the comparison of the effect of both of them (**C**) for 1h. Same analysis was carried out at 2h (**D**–**F**). Each measurement was performed in triplicate. Error bars = standard deviation (n = 3). Statistically significant differences were calculated using one-way ANOVA test (*p < 0.05; **p < 0.01; ***p < 0.001; **** p < 0.0001).

concentrations. At 60% contrast, IB significantly enhanced cell viability compared to GA. hNP cells demonstrated higher viability than hAF cells at higher concentrations. Regarding to the influence of the type of PRP, BPCP significantly improved cell viability compared to sPRP at high contrast concentration levels. These findings highlight the importance of contrast concentration, exposure duration, and PRP formulations in influencing cell viability.

Discussion

The main findings of this work showed that GA and IB both could induce cytotoxicity in hAF and hNP in a dose- and time-dependent manner. Nevertheless, PRP supplementation significantly could enhanced cell viability, especially with BPCP at higher contrast agent concentrations. Moreover, multivariate regression analyses identified contrast concentration, exposure time, and PRP formulation as possible critical factors influencing cell viability. The trend observed in these preliminary results opens up a new line of research to address the problems of contrast use in clinical IVD, underscoring the need to minimize contrast agent exposure during IVD procedures. Moreover, these findings suggest that BPCP may provide enhanced protection against contrast-induced cytotoxicity. These findings also could provide important insights into optimizing the use of contrast agents in IVD interventions.

Regarding the overall effects of the key variables on cell viability, both contrast agent concentration and exposure time could be strongly associated with reduced cell viability. These findings emphasize a possible cytotoxicity of contrast agents is dose- and time-dependent. Interestingly, the contrast agents' cytotoxic effects were comparable between hAF and hNP cells under the tested conditions, although at high contrast concentrations hAF cells were more affected in the performed assay. In contrast, the addition of PRP significantly improved cell viability, suggesting its protective role regardless of the experimental conditions.

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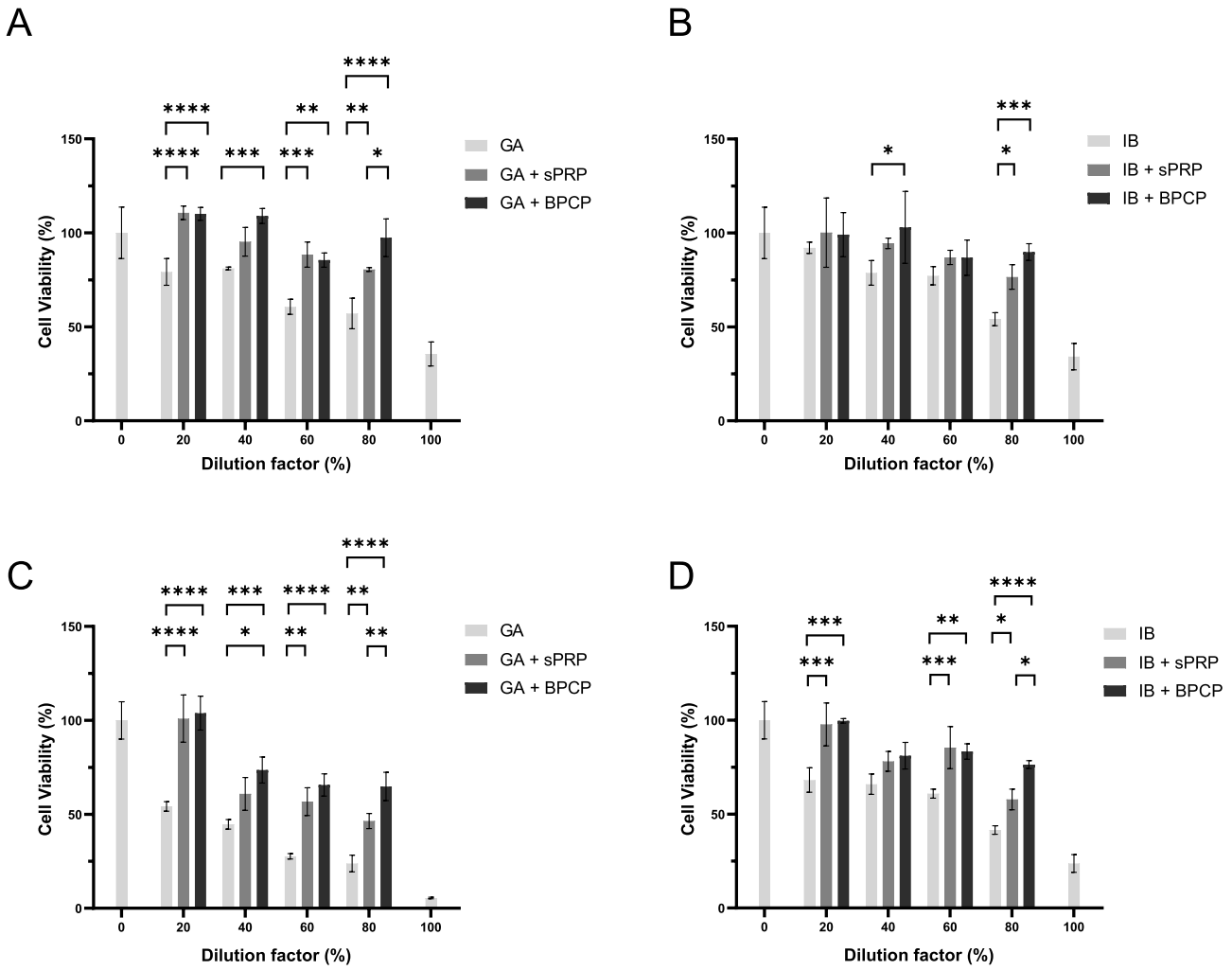

Figure 5 Cell viability percentage of hNP cells exposed to different concentrations of gadoteric acid (GA) and iobitridol (IB) combined both wit sPRP or BPCP. Measurement of cell viability of hNP exposed to GA (**A**) and IB (**B**) at different concentrations combined with 10% sPRP or BPCP supplemented cell medium for 1h. Same analysis was carried out at 2h (**C** and **D**). Each measurement was performed in triplicate. Error bars = standard deviation (n = 1). Statistically significant differences were calculated using two-way ANOVA test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

The differential effects observed between GA and IB align with previous findings regarding their biocompatibility. GA and IB exhibited a cytotoxic profile, which could make it an unsuitable choice for procedures requiring higher concentrations or extended exposure times. Exposure time (1h vs 2h) consistently appeared to show a strong negative

Table 2 Multivariate Regression Analysis for Cell Viability

	B	Beta	*p* value	98.8% CI		VIF
Concentration	−132.31	−0.67	***0.001*****	−148.47	−116.16	1.021
Exposure time ($1h^R$/2h)	−12.69	−0.23	***0.001*****	−17.24	−8.16	1.010
Cell line (hAF^R/hNP)	3.40	0.06	*0.058*	−1.12	7.92	1.001
PRP (No^R/Yes)	23.34	0.41	***0.001*****	18.68	28.00	1.015

Notes: R = reference category. Numbers in bold represent statistically significance: ***p<0.001. N (observations) = 3 triplicates × 1 pooled sample per condition. $R^2 = 0.693$. The regression model includes four main predictors. Each condition was measured in triplicate from a single pooled biological sample from 5 donors. The reported n corresponds to the number of technical replicates included in the analysis. As only a single biological sample was used, these results should be interpreted as exploratory and descriptive.

Abbreviations: B, unstandardized coefficient; Beta, standardized coefficient; CI, confidence interval; hAF, human anulus fibrosus; hNP, human nucleus pulposus; PRP, Platelet-Rich Plasma; VIF, Variance Inflation Factors.

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Table 3 Multivariate Regression Analysis for Cell Viability at Different Concentrations

	B	Beta	*p* value	98.8% CI		VIF
20% contrast concentration						
Contrast type (GA^R/IB)	−6.22	−0.20	*0.156*	−17.57	5.15	1.006
Exposure time ($1h^R$/2h)	−14.24	−0.46	***0.002*** **	−25.57	−2.91	1.007
Cell line (hAF^R/hNP)	2.91	0.09	*0.503*	−8.45	14.26	1.006
PRP type ($sPRP^R$/BPCP)	−6.73	0.22	*0.125*	−18.06	4.59	1.007
40% contrast concentration						
Contrast type (GA^R/IB)	4.92	0.15	*0.220*	−5.49	15.34	1.006
Exposure time ($1h^R$/2h)	−21.83	−0.64	***0.001*** ***	−32.25	−11.42	1.006
Cell line (hAF^R/hNP)	2.34	0.07	*0.558*	−8.08	12.75	1.006
PRP type ($sPRP^R$/BPCP)	−5.70	−0.17	*0.158*	−16.12	4.71	1.006
60% contrast concentration						
Contrast type (GA^R/IB)	15.45	0.53	***0.001*** ***	7.51	23.39	1.002
Exposure time ($1h^R$/2h)	−13.50	−0.47	***0.001*** ***	−21.46	−5.54	1.007
Cell line (hAF^R/hNP)	8.04	0.28	***0.011*** *	0.11	15.99	1.002
PRP type ($sPRP^R$/BPCP)	−1.31	−0.05	*0.667*	−9.25	6.64	1.006
80% contrast concentration						
Contrast type (GA^R/IB)	−7.09	−0.21	*0.046*	−16.14	1.97	1.002
Exposure time ($1h^R$/2h)	−18.15	−0.53	***0.001*** ***	−27.19	−9.09	1.002
Cell line (hAF^R/hNP)	13.97	0.41	***0.001*** ***	4.92	23.01	1.002
PRP type ($sPRP^R$/BPCP)	11.31	0.33	***0.002*** **	2.29	20.36	1.002

Notes: R = reference category. Numbers in bold represent statistically significance: *p<0.0125; **p<0.01; ***p<0.001. N (observations) = 3 triplicates × 1 pooled sample per condition. R^2 (20%) = 0.319; R^2 (40%) = 0.461; R^2 (60%) = 0.548; R^2 (80%) = 0.583. The regression model includes four main predictors. Each condition was measured in triplicate from a single pooled biological sample from 5 donors. The reported n corresponds to the number of technical replicates included in the analysis. As only a single biological sample was used, these results should be interpreted as exploratory and descriptive.

Abbreviations: B, unstandardized coefficient; Beta, standardized coefficient; CI, confidence interval; hAF, human anulus fibrosus; hNP, human nucleus pulposus; PRP, platelet-rich plasma; sPRP, standard PRP; BPCP, balanced protein-concentrate plasma; VIF, Variance Inflation Factors.

association with cell viability across all concentrations, obtaining higher cell viability with 1h of contact. These observations suggest that shorter contact times between contrast agents and IVD cells may be associated with reduced cytotoxic effects.

With respect to IB, there is lack of research on its mechanism of toxicity. However, different studies with other iodinated contrasts have been published. In a study carried out by Chee AV et al[32] it was found that the iodinated contrast agents Iohexol and Iopamidol were toxic in hNP and hAF cells. In agreement with our results, hAF cells were found to be more sensitive to the toxic effects of iodine contrast agents. Therefore, this suggests that the structural and functional differences between these cell types may influence their vulnerability to chemical exposure.[32] In the study, they found that toxicity was also dose- and time-dependent, too. Nevertheless, in studies performed with other iodinated contrasts, different results were obtained. For example, Gradisnik et al analyze the toxicity of Iodixanol and they concluded that it affected hNP cells more than hAF cells.[36] In turn, Iwasaki K. et al described that another iodinated contrast, Iotrolan, was not toxic in hNP cells.[37] Therefore, this indicates that the toxicity of iodinated contrasts in IVD depends on the type of contrast. Moreover, it has been reported that the safety profiles of individual iodinated contrast media are significantly different.[38] The toxicity of iodinated contrast media in human disc cells has been classified according to the type of contrast used. In the study conducted by Kim KH. et al they determined that the highest toxicity was shown by ionic monomeric contrasts, followed by ionic dimeric, non-ionic monomeric and finally non-ionic dimeric contrasts.[39]

Regarding GA contrast toxicity, it is primarily linked to its deposition in various tissues, including the skin, brain, and kidneys. The toxicity mechanisms involve interference with calcium homeostasis and mitochondrial functions, leading to

cellular damage.[40,41] Gadolinium can induce mitochondrial toxicity and cell death in human neurons, with increased toxicity observed in agents with lower kinetic stability.[40] GA chelates (Gd^{3+}) are widely used as contrast media for magnetic resonance imaging. Depending on the chemical structure, GA based contrast agents are classified as linear or macrocyclic and ionic or non-ionic.[33–35] Although there are different clinically accepted drugs, in recent years different toxic effects have been described in different tissues such as brain, kidneys, skin and bones.[42] One of the reasons may be the accumulation of GA in these tissues due to a lack of gadolinium clearance. GA accumulation can lead, among other things, to fibrosis, bone and joint pain and skin thickening.[42–45]

GA based contrast agents toxicity is related to unchelated ion gadolinium, which is present in all molecules.[44] To date, different molecular mechanisms of toxicity have been described in different cell types such as neurons, macrophages, fibroblasts and hepatocytes, among others. Of the molecular mechanisms, those involving interference with calcium homeostasis and mitochondrial functions stand out which lead to cellular damage.[34–36,38,39] For example, Wermuth and Jimenez et al[46] described that GA induced the of expression of a profibrotic chemokines and cytokines such as IL-4, IL-6, IL-13, and VEGF in monocytes and type I and II collagen in fibroblasts. Moreover, Xia et al[47] described the elevation of reactive oxygen species in mitochondria exposed to GA.

In terms of PRP type, the data suggest that co-administration of PRP with contrast agents may be associated with increased cell viability. At higher contrast concentrations (eg, 80%), the type of PRP (sPRP vs BPCP) appeared to influence this effect, with BPCP showing the highest observed cell viability. The protective role of PRP was evident across all experimental conditions and concentrations. Its ability to mitigate the adverse effects of contrast agents is likely attributed to its rich composition of GFs, anti-inflammatory cytokines, and other bioactive molecules that support cellular repair and survival. Kim H-J. et al demonstrated that PRP had anti-inflammatory effects on hNP cells, particularly in response to pro-inflammatory cytokines such as TNF-α and IL-1. Moreover, they evidenced that PRP exposure can significantly recover the expression of matrix synthesis genes like collagen type II and aggrecan, while reducing the expression of degradation genes such as MMP-3 and COX-2.[48] This suggests that PRP can stabilize hNP cell differentiation and counteract inflammation-induced degradation. Hu B. et al[49] studied the effect of microRNA miR-25-3p carried by extracellular vesicles derived from PRP, which plays a role in alleviating hNP cell degeneration. miR-25-3p inhibits the expression of SOX4 and limits CXCR7 transcription, thereby reducing cell apoptosis and inflammation in IL-1β-induced hNP cells. This mechanism highlights the potential of PRP in mitigating cellular degeneration and promoting tissue repair.

Regarding hAF cells, PRP contains cytokines and GFs, such as transforming TGF-β and PDGF, which can reduce inflammation in hAF cells. These factors may downregulate inflammatory pathways, thereby decreasing the expression of inflammatory mediators.[50] GFs present in PRP, including VEGF and epidermal growth factor (EGF), enhance angiogenesis and cellular proliferation. These factors support hAF cell recovery by stimulating extracellular matrix synthesis, particularly collagen and proteoglycans, which are critical for maintaining disc integrity.[23] PRP may upregulate anti-apoptotic proteins and inhibit pro-apoptotic pathways, preserving the cell population and functional capacity of the hAF.[51] Finally, PRP can influence the activity of MMPs, enzymes that degrade the ECM, thereby preserving the structural integrity of the hAF.[25] These effects of PRP may explain its clinical efficacy in the treatment of IVD pathology.[52]

Notably, the observed enhanced effects of BPCP at higher contrast concentrations suggest that PRP formulations with optimized GF profiles may provide superior protection. Indeed, the increased levels of extra platelet molecules compared to sPRP could be related with the possible protective role of BPCP in IVD. For example, IGF-1, HGF and exosomes has been shown to have protective effects on hNP and hAF cells. Several studies demonstrated that IGF-1 promotes cell proliferation, reduces inflammation and inhibits apoptosis, contributing to the maintenance of IVD homeostasis and potentially mitigating disc degeneration.[53–55] Same results were described in different studies regarding HGF, which promotes cell proliferation and enhances the expression of extracellular matrix components in hNP cells, which are crucial for maintaining the structural integrity of intervertebral discs.[56,57] Moreover, several GF synergic effects were described.[58,59] Finally, regarding exosomes, protective effect against cell degeneration was attributed to their capacity for inhibit apoptosis,[60–62] reduce oxidative stress,[62,63] inflammation[63,64] and matrix synthesis,[62,65] especially those derived from mesenchymal stem cells (MSCs) and PRP.[66]

The results of this study suggest possible clinical relevance, although additional studies are needed to establish their significance. First, the data implied a potential benefit in minimize both the concentration and exposure duration of contrast agents during imaging-guided interventions. Second, the co-administration of PRP appeared to be associated with improved cell viability, indicating a possible strategy to enhance the safety and efficacy of these procedures. The application of PRP following discography may help reducing the presence of contrast agent and could ssuport the maintenance of a favorable cellular environment in the hNP, as well as contribute to the integrity of the hAF after puncture. Moreover, BPCP showed a trend toward higher cell viability compared with sPRP in the conditions tested. Finally, differences observed between contrast agents indicate that their cytotoxic profiles could be considered when evaluating experimental outcomes, although further studies are required to asses clinical relevance.

However, the present study has several important limitations that should be acknowledged more explicitly. The use of high contrast fractions in vitro may not fully reflect physiological conditions. Moreover, the concentration of contrast agent in vitro does not reflect the behavior of GA or IB intervertebral disc in vivo, due to the lack of studies that describe the diffusion of the contrast in the organs as the use two-dimensional culture system lacks the structural and functional complexity of in vivo tissues. An additional limitation was the use of pooled plasma from five donors, which, although it reduces inter-individual variability, was necessary due to the complexity of the experimental procedure and consequently limits the ability to assess donor-specific responses. Nethertheless, as one biological sample was used, it could be necessary to increase the biological replicate samples. Another important limitation was the absence of critical controls required to assess potential confounding effects related to osmolality, pH, apoptosis, and necrosis, which restricted the ability to accurately attribute the observed cellular responses to the treatments administered. Moreover, molecular analysis of GA and IB should be carried out with the aim of describe molecular mechanism of toxicity in IVD cells. Due to the different biochemical and biological characteristics of the contracting agents, it would be interesting to test the combination of PRP with other agents used in the market. It would be interesting to analyze the effect of individual extraplatelet molecules in combination with contrast agents to determine the relevance in the improvement of cell survival, too. Finally, another complementary assays would be done such as washout experiments, three dimensional or explant models, donor level analyses, basic mechanism assays on mitochondrial function and calcium balance, in addition to in vivo validation. All these limitations and analysis proposals could be carried out in future studies in order to perform a more complete study of the characterization GA and IB toxicity in IVD cells and the use of BPCP in combination with them to reduce cell toxicity, facilitating the transition from the experimental to the clinical part.

Conclusions

This study demonstrates the possible significant in vitro cytotoxic effects of GA and IB on hAF and hNP cells, with both dose and exposure time playing crucial roles in reducing cell viability. The data suggest a feasible higher viability under exposure tendency of PRP, particularly the BPCP formulation, in mitigating these cytotoxic effects, especially at higher contrast concentrations. The findings underscore the importance of optimizing contrast agent usage during IVD procedures by minimizing both concentration and exposure duration. Moreover, the inclusion of PRP formulations could serve as a promising protective adjunct to improve cell viability and potentially enhance IVD regeneration Nevertheless, further in vitro and in vivo studies are required to determine the clinical relevance of these findings.

Study Novelty Statement

The present study addresses the possible negative effect of contrast agents on intervertebral disc cell populations. The protective effect of PRP against such substances is also evaluated. Furthermore, not only the effect of standard PRP is analyzed but also that of a new type of PRP called BPCP (Balanced Protein-Concentrate Plasma) whose characteristics and general effects have been previously studied (references 15 and 28). In this study we analyze for the first time this product on annulus fibrosus and nucleus pulposus populations and its differences in biological and protective effect in comparison with conventional PRP.

Institutional Review Board Statement

This study was in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of the OSI Araba (2024-023, 30 May 2024).

Abbreviations

AF, Annulus Fibrosus; BPCP, Balanced Protein-Concentrate Plasma; ECM, Extracellular Matrix; FBS, Fetal Bovine Serum; GA, Gadoteric Acid; GF, Growth Factor; hAF, Human Annulus Fibrosus; HGF, Hepatocyte Growth Factor; hNP, Human Annulus Fibrosus; IB, Iobitridol; IGF-1, Insulin-like Growth Factor; IVD, Intervertebral Disc; MRI, Magnetic Resonance Imaging; NP, Nucleus Pulposus; PDGF, Platelet-derived Growth Factor; PRP, Platelet-rich Plasma; RT, Room Temperature; sPRP, Standard Platelet-rich Plasma; TGF-β1, Transforming Growth Factor Beta 1; VEGF, Vascular Endothelial Growth Factor.

Data Sharing Statement

Data will be made available on request.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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