Klinik für Mund-, Kiefer- und Gesichtschirurgie – Plastische Operationen
der Universitätsmedizin der Johannes-Gutenberg-Universität Mainz

Kombination verschiedener Knochenersatzmaterialien mit injizierbarem plättchenreichem Fibrin – vergleichende in vitro-Studien

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Zusammenfassung (deutsch)

Ziel der in vitro-Studien war ein Vergleich des Effekts verschiedener Knochenersatzmaterialien (allogen, (AKEM) und xenogen (XKEM)) mit und ohne injizierbarem plättchenreichem Fibrin (i-PRF) auf verschiedene Zelleigenschaften humaner Osteoblasten (HOB). Es wurden zu einem ein AKEM und ein bovines XKEM und zum anderen vier bovine XKEM (Cerabone® (CB), Bio-Oss® (BO), Creos Xenogain® (CX) und MinerOss® X (MO)) für 3, 7 und 10 Tage mit HOB mit und ohne i-PRF (+ i-PRF = Test; - i-PRF = Kontrolle) inkubiert. Zellvitalität, -migration, -proliferation und -differenzierung (alkalische Phosphatase (AP), knochenmorphogenetisches Protein 2 (BMP-2) und Osteonectin (OCN)) wurden gemessen und zwischen den Gruppen verglichen.

In der ersten in vitro-Studie, in der AKEM mit XKEM verglichen wurde, wurde an Tag 3 für AKEM-i-PRF eine erhöhte zelluläre Vitalität, Migration und Proliferation beobachtet. Für Vitalität und Proliferation (Tag 7 und 10) sowie für Migration (Tag 10) zeigte AKEM-i-PRF/XKEM-i-PRF höhere Werte im Vergleich zu AKEM/XKEM mit Maximalwerten für AKEM-i-PRF und Minimalwerten für XKEM. An den Tagen 3 und 7 wurde die höchste Expression von AP in AKEM-i-PRF/XKEM-i-PRF im Vergleich zu AKEM/XKEM nachgewiesen, während an Tag 10 die AP-Expressionsspiegel in AKEM-i-PRF/AKEM erhöht waren. Die höchste BMP-2-Expression wurde in AKEM-i-PRF beobachtet, während die OCN-Expression höhere Spiegel in AKEM-i-PRF/XKEM-i-PRF an den Tagen 3 und 7 mit der niedrigsten Expression für AKEM zeigte. Später wurden nur bei AKEM-i-PRF erhöhte OC-Werte festgestellt. Zusammenfassend lässt sich sagen, dass i-PRF in Kombination mit AKEM die HOB-Aktivität im Vergleich zu XKEM-i-PRF oder unbehandeltem KEM *in vitro* verbessert. Daher kann die Zugabe von i-PRF zu AKEM und – in geringerem Maße – zu XKEM die Osteoblastenaktivität *in vivo* beeinflussen.

In der zweiten *in vitro*-Studie, bei der vier bovine XKEM verglichen wurden, wurden in der nicht-i-PRF-Gruppe für CB zu allen Zeitpunkten die höchsten Werte bezüglich der Zellvitalität beobachtet. Die Vorbehandlung mit i-PRF erhöhte die Vitalität in allen Gruppen mit den höchsten Werten für CB-i-PRF nach 3 und 7 und für CX-i-PRF nach 10 Tagen. Bei der metabolischen Aktivität wurde die höchste Rate in der nicht-i-PRF-Gruppe für MO an Tag 3 und für CB an Tag 7 und 10 beobachtet. Auch hier zeigten i-PRF-XKEM höhere Werte als die die XKEM ohne i-PRF (höchste Werte: CB-i-PRF) zu allen Zeitpunkten. Es gab keinen Unterschied in der ALP-Expression zwischen den Gruppen. Für die OCN-Expression in der nicht-i-PRF-Gruppe zeigte CB die höchsten Werte an Tag 3, CX an Tag 7 und 10. In der i-PRF-Gruppe wurden die höchsten Werte für CX-i-PRF beobachtet. An Tag 3 wurde die höchste BMP-2-Expression für CX mit dem höchsten Anstieg für CX-i-PRF an Tag 3 beobachtet. An Tag 7 und 10 gab es keinen signifikanten Unterschied zwischen den Gruppen. Somit zeigten unter hohen Temperaturen gesinterte XKEM im Vergleich zu XKEM, die bei niedrigeren Temperaturen hergestellt wurde, eine erhöhte HOB-Vitalität und metabolische

Aktivität während des gesamten Zeitraums. Insgesamt verbesserte auch hier die Kombination von XKEM mit i-PRF alle zellulären Parameter, die ALP- und BMP-2-Expression in früheren Stadien sowie die OCN-Expression in späteren Stadien.

Zusammenfassung (englisch)

The aim of the in vitro study was a comparison of different bone substitute materials (allogenic, (ABSM) and xenogenic (XBSM) with and without injectable platelet-rich fibrin (ABSM-i-PRF & XBSM-i-PRF) on cell characteristics of human osteoblasts (HOB). Here, ABSM and XBSM in one publication and four bovine XBSM (Cerabone® (CB), Bio-Oss® (BO), Creos Xenogain® (CX) and MinerOss® X (MO)) with and without i-PRF (+ i-PRF = test; - i –PRF = control) were incubated with HOB for 3, 7 and 10 days. HOB viability, migration, proliferation and differentiation (RT-PCR on alkaline phosphatase (AP), bone morphogenetic protein 2 (BMP-2) and osteonectin (OCN)) were measured and compared between groups.

In the first *in vitro* study, in which ABSM was compared with XBSM, at day 3, an increased viability, migration and proliferation was seen for ABSM-i-PRF. For viability and proliferation (days 7 and 10) and for migration (day 10), ABSM-i-PRF/XBSM-i-PRF showed higher values compared to ABSM/XBSM with maximum values for ABSM-i-PRF and minimum values for XBSM. At days 3 and 7, the highest expression of AP was detected in ABSM-i-PRF/XBSM-i-PRF when compared to ABSM/XBSM, whereas at day 10, AP expression levels were elevated in ABSM-i-PRF/ABSM. The highest BMP-2 expression was seen in ABSM-i-PRF whereas OCN expression showed higher levels in ABSM-i-PRF/ XBSM-i-PRF at days 3 and 7 with lowest expression for ABSM. Later on, elevated OC levels were detected for ABSM-i-PRF only. In conclusion, i-PRF in combination with ABSM enhances HOB activity when compared to XBSM-i-PRF or untreated BSM in vitro. Therefore, addition of i-PRF to ABSM and –to a lower extent –to XBSM may influence osteoblast activity in vivo.

In the second in vitro study, in which four bovine bone XBSM were compared, for non-i-PRF groups, the highest values concerning viability were seen for CB at all time points. Pre-treatment with i-PRF increased viability in all groups with the highest values for CB-i-PRF after 3 and 7 and for CX-i-PRF after 10 days. For metabolic activity, the highest rate among the non-i-PRF group was seen for MO at day 3 and for CB at day 7 and 10. Here, the i-PRF group showed higher values than non-i-PRF groups (highest values: CB-i-PRF) at all time points. There was no difference in ALP expression between groups. For OCN expression in non-i-PRF groups, CB showed the highest values after day 3, CX after day 7 and 10. Among i-PRF-groups, the highest values were seen for CX-i-PRF. At day 3, the highest BMP-2 expression was observed for CX and the highest increase was seen for CX-i-PRF at day 3. At day 7 and 10, there was no significant difference among groups. In conclusion, XBSM sintered under high temperature showed an increased HOB viability and metabolic activity through the whole period when compared to XBSM manufactured at lower temperatures. Overall, the combination of XBSM with i-PRF improved all cellular parameters, ALP and BMP-2 expression at earlier stages as well as OCN expression at later stages.

Abkürzungsverzeichnis

Deutsch

AKEM – allogenes Knochenersatzmaterial

XKEM – xenogenes Knochenersatzmaterial

i-PRF – injizierbares plättchenreiches Fibrin

HOB – humane Osteoblasten

CB – Cerabone®

BO – Bio-Oss®

CX – Creos Xenogain®

MO – MinerOss® X

AP – Alkaline Phosphatase

BMP-2 – knochenmorphogenetisches Protein 2

OCN – Osteonectin

English

ABSM – allogenic bone substitute material

XBSM – xenogenic bone substitute material

i-PRF – injectable platelet-rich fibrin

HOB – human osteoblasts

CB – Cerabone®

BO – Bio-Oss®

CX – Creos Xenogain®

MO – MinerOss® X

AP – alkaline phosphatase

BMP-2 – bone morphogenetic protein 2

OCN – osteonectin

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1 Einleitung / Ziel der Dissertation

For osseous regeneration, autologous bone is considered to be the biological gold standard in regard of its osteogenic, osteoconductive and osteoinductive properties. Whether or not autologous bone can be considered as the clinical gold standard in all cases can be discussed controversially as it entails a number of limitations, i.e. the need for an additional operative donor site with a higher morbidity and sometimes rapid resorption of the grafts. A clinical alternative to autologous bone may represent bone substitute materials (BSM) that are widely used in regenerative medicine as a valid approach for osseous reconstruction and each BSM has its advantages as well as limitations and disadvantages. Most frequently used BSM are of allogenic (ABSM), xenogenic (XBSM), or synthetic origin [1, 2].

For ABSM, in addition to its unlimited availability, the possibility of patient-specific customization and its biocompatibility as well as decent osteoinductive properties are reported [3]. For XBSM, mostly of bovine origin, the main effects – osteoconduction and a low absorbability rate – were described in detail [4]. It was also reported that a biomimetic modified xenograft could enhance angiogenesis and osteogenesis [5]. Mardinger et al. stated that xenografts have almost no changes or no changes in bone height in comparison to autogenous bone or a combination of both [6]. Additionally, the composition of bovine bone substitutes is similar to human bone due to the preserved microstructure of the osseous frame [7, 8]. Deproteinization potentially allows elimination of transmission risk and antigenicity [9]. However, different cleaning and manufacturing methods may affect the regeneration capacity of the bovine bone substitute material. For example, manufacturing of deproteinized bovine bone by sintering consists of high temperature treatment with stepwise heating up to > 1,000°C leading to the removal of all organic components including collagen [9]. On contrary, manufacturing with lower temperatures usually comprise an additional chemical treatment, i.e., with sodium hydroxide with efficiently inactivated viruses [10]. Thus, Cerabone® (Botiss, Zossen, Germany) is produced via three-stage temperature treatment including a final sintering at > 1,200°C, hence all organic compounds are removed and potential prions, bacteria and viruses are eliminated. This preparation process might alter the microstructure [11]. However, it has been shown that Cerabone® resembles the structure of natural bone with high porosity and rough surface [12]. Bio-Oss® (Geistlich Pharma AG, Wolhusen, Switzerland) has a fiber-like surface with a much smaller crystal size [11, 13]. It is manufactured at a lower temperature of 300°C followed by sodium hydroxide treatment [14]; thus, it is considered to be a hydroxyapatite ceramic with a high porosity including large interconnective pores and residual proteins [14] Klein [15]. Creos Xenogain® (Nobel Biocare GmbH, Gothenburg, Sweden) is produced by sodium hypochloride treatment followed by heating under 400°C. MinerOss® X (BioHorizons, Birmingham, United Kingdom) is also produced via low-heat processing of bovine bone, preserving the coarseness of bone with a high porosity.

To enhance tissue regeneration, autologous platelet concentrates have been developed and successfully used in the clinical setting [16, 17]. Besides, platelets were found to enhance bone healing in healing-impaired cases by promoting early cellular proliferation leading to better qualitatively and quantitatively bone formation [18, 19]. Here, platelet-rich plasma (PRP) has been produced using a specific centrifugation protocol together with anti-coagulants. A number of studies reported a positive effect of PRP in combination with BSM on human osteoblasts (HOB) in vitro [15, 20]; however, others contradict it [21-23]. Additionally, regenerative capacity of PRP was reported to be short and anti-coagulants needed for preparation of PRP seemed to inhibit wound healing processes [22, 24, 25]. Platelet-rich fibrin (PRF) is a leucocyte-and platelet rich fibrin matrix, a further development of PRP and therefore second generation of platelet concentrates as proposed by Choukroun et al. [26]. Specific centrifugation pattern after blood harvest from a 10 ml blood sample enables PRF to build up a three-dimensional matrix of platelets and leukocytes [27]. Due to the fibrin component, PRF acts as a scaffold that triggers cell–cell contacts and proliferation [28]. Besides, PRF does not dissolve quickly after application that favors a gradual release of growth factors [29] for at least seven days [30]. Subsequently, the suitability of PRF as a biologically active scaffold has been illustrated proving a higher proliferation and differentiation rate of HOB and gingival fibroblasts [31-33]. There is also emerging evidence that PRF may increase HOB migration and proliferation in vitro [26, 30, 31]. Additionally, it has been demonstrated that PRF promotes angiogenesis [34]. Furthermore, among its advantages are also simple preparation without blood modification, ease of handling, and minimal expense [35]. Patients' blood is collected through venipuncture and immediately centrifuged without addition of an anticoagulant, carrier, or activator [36], leading to activation of the physiological coagulation cascade [37, 38]. Recently, a lower centrifugation protocol of PRF production, so called injectable-PRF (i-PRF) was developed [39]. I-PRF remains liquid for approximately 15 min. After application, it immediately coagulates into a PRF clot rich with blood-derived growth factors, which constantly release over 10–14 days [40]. I-PRF has advantage in higher number of leukocyte as far as it favors more growth factor release and thus tissue wound healing [41]. Although i-PRF is widely used alone and as a carrier for variety of biomolecules in medicine, its potential in combination with variety biomaterials still lacks scientific evidence [42]. Angiogenesis and osteogenesis are coupled in the process of osteogenesis [43]. BSM are routinely used in maxillofacial surgery as a valid alternative to the autologous bone as the gold standard when bone is lost due to trauma, cancer, or other pathologies [44]. Therefore, the combination of BSM with platelet concentrates such as i-PRF may represent a clinical pathway to enhance angiogenesis and therefore bone repair.

1.1

The aim of this preclinical in vitro study was to compare the effect of different BSM with and without additional i-PRF on HOB viability, attachment, proliferation, and differentiation. The null hypothesis was that different BSM in combination with i-PRF may improve proliferation, cell attachment and differentiation of osteoblasts when compared to BSM without i-PRF.

2 Publizierte Originalarbeiten

1. Combination of an allogenic and a xenogenic bone substitute material with injectable platelet-rich fibrin–A comparative in vitro study
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Combination of an allogenic and a xenogenic bone substitute material with injectable platelet-rich fibrin – A comparative in vitro study

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Abstract
The aim of the in vitro study was a comparison of an allogenic (ABSM) and a xenogenic bone substitute material (XBSM) with and without injectable platelet-rich fibrin (ABSM-i-PRF & XBSM-i-PRF) on cell characteristics of human osteoblasts (HOB). Here, ABSM and XBSM (þ i-PRF¼test; - i-PRF¼control) were incubated with HOB for 3, 7 and 10 days. HOB viability, migration, proliferation and differentiation (RT-PCR on alkaline phosphatase (AP), bone morphogenetic protein 2 (BMP-2) and osteonectin (OCN)) were measured and compared between groups. At day 3, an increased viability, migration and proliferation was seen for ABSM-i-PRF. For viability and proliferation (days 7 and 10) and for migration (day 10), ABSM-i-PRF/XBSM-i-PRF showed higher values compared to ABSM/XBSM with maximum values for ABSM-i-PRF and minimum values for XBSM. At days 3 and 7, the highest expression of AP was detected in ABSM-i-PRF/XBSM-i-PRF when compared to ABSM/XBSM, whereas at day 10, AP expression levels were elevated in ABSM-i-PRF/ABSM. The highest BMP-2 expression was seen in ABSM-i-PRF whereas OCN expression showed higher levels in ABSM-i-PRF/XBSM-i-PRF at days 3 and 7 with lowest expression for ABSM. Later on, elevated OC levels were detected for ABSM-i-PRF only. In conclusion, i-PRF in combination with ABSM enhances HOB activity when compared to XBSM-i-PRF or untreated BSM in vitro. Therefore, addition of i-PRF to ABSM and – to a lower extent – to XBSM may influence osteoblast activity in vivo.

Keywords
Allogenic bone, xenogenic bone, injectable platelet-rich fibrin, bone substitute material, osteoblast activity, in vitro

Introduction

For osseous regeneration, autologous bone is considered to be the biological gold standard in regard of its osteogenic, osteoconductive and osteoinductive properties. Whether or not autologous bone can be considered as the clinical gold standard in all cases can be discussed controversially as it entails a number of limitations, i.e. the need for an additional operative donor site with a higher morbidity and sometimes rapid resorption of the grafts. A clinical alternative to autologous bone may represent bone substitute materials (BSM) that are widely used in regenerative medicine as a valid approach for osseous reconstruction and each BSM has its advantages as well as limitations and disadvantages. Most frequently used BSM are of allogenic (ABSM), xenogenic (XBSM), or synthetic origin.[1,2] For XBSM, mostly of bovine origin, the main effects – osteoconduction and a low absorbability rate – were described in detail.[3] It was also reported that a biomimetic modified xenograft could enhance angiogenesis

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and osteogenesis.[4] Mardinger et al. stated that xenografts have almost no changes or no changes in bone height in comparison to autogenous bone or a combination of both.[5] For ABSM, in addition to its unlimited availability, the possibility of patient-specific customization and its biocompatibility as well as decent osteoinductive properties are reported.[6]

To enhance tissue regeneration, autologous platelet concentrates have been developed and successfully used in the clinical setting.[7,8] Besides, platelets were found to enhance bone healing in healing-impaired cases by promoting early cellular proliferation leading to better qualitatively and quantitatively bone formation.[9,10] Here, platelet-rich plasma (PRP) has been produced using a specific centrifugation protocol together with anti-coagulants. A number of studies reported a positive effect of PRP in combination with BSM on human osteoblasts (HOB) in vitro;[11,12] however, others contradict it.[13–15] Additionally, regenerative capacity of PRP was reported to be short and anti-coagulants needed for preparation of PRP seemed to inhibit wound healing processes.[14,16,17]

Platelet-rich fibrin (PRF) is a leucocyte- and platelet-rich fibrin matrix, a further development of PRP and therefore second generation of platelet concentrates as proposed by Choukroun et al.[18] Specific centrifugation pattern after blood harvest from a 10 ml blood sample enables PRF to build up a three-dimensional matrix of platelets and leukocytes.[19] Due to the fibrin component, PRF acts as a scaffold that triggers cell–cell contacts and proliferation.[20] Besides, PRF does not dissolve quickly after application that favors a gradual release of growth factors[21] for at least seven days.[22]

Subsequently, the suitability of PRF as a biologically active scaffold has been illustrated proving a higher proliferation and differentiation rate of HOB and gingival fibroblasts.[23–25] There is also emerging evidence that PRF may increase HOB migration and proliferation in vitro.[18,23,26] Additionally, it has been demonstrated that PRF promotes angiogenesis.[27] Furthermore, among its advantages are also simple preparation without blood modification, ease of handling, and minimal expense.[28] Patients' blood is collected through venipuncture and immediately centrifuged without addition of an anticoagulant, carrier, or activator,[29] leading to activation of the physiological coagulation cascade.[30,31]

Recently, a lower centrifugation protocol of PRF production, so called injectable-PRF (i-PRF) was developed.[32] I-PRF remains liquid for approximately 15 min. After application, it immediately coagulates into a PRF clot rich with blood-derived growth factors, which constantly release over 10–14 days.[33] I-PRF has advantage in higher number of leukocyte as far as it favors more growth factor release and thus tissue wound healing.[34] Although i-PRF is widely used alone and as a carrier for variety of biomolecules in medicine, its potential in combination with variety biomaterials still lacks scientific evidence.[35]

Angiogenesis and osteogenesis are coupled in the process of osteogenesis.[36] BSM are routinely used in maxillofacial surgery as a valid alternative to the autologous bone as the gold standard when bone is lost due to trauma, cancer, or other pathologies.[37] Therefore, the combination of BSM with platelet concentrates such as i-PRF may represent a clinical pathway to enhance angiogenesis and therefore bone repair.

Therefore, the aim of this preclinical in vitro study was to compare the effect of allogenic and xenogenic BSM with and without additional i-PRF on HOB viability, attachment, proliferation, and differentiation. The null hypothesis was that allogenic and xenogenic BSM in combination with i-PRF may improve proliferation, cell attachment and differentiation of osteoblasts when compared to both BSM without i-PRF.

Materials and methods

In the current study, allogenic (ABSM; maxgraft®, botiss biomaterials GmbH, Zossen, Germany, granularity < 2 mm) and xenogenic BSM (XBSM; BioOss®, Geistlich Pharma AG, Wolhusen, Switzerland, granularity 1–2 mm) were used.

A commercially available human osteoblast cell line (HOB, PromoCell, Heidelberg, Germany) was applied. For cultivation, a standard HOB medium was administered, which contained fetal calf serum (FCS, Gibco Invitrogen, Karlsruhe, Germany), Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen), dexamethasone (100 nmol/l, Serva Bioproducts, Heidelberg, Germany), L-glutamine (Gibco Invitrogen), and streptomycin (100 mg/ml, Gibco Invitrogen). Cultivation of HOB was conducted at 37°C in a constant, humidified atmosphere with 95% room air and 5% CO_2. The passaging of HOB was conducted on demand of their growth state (70% confluence), using 0.25% trypsin (Seromed Biochrom KG, Berlin, Germany).

Peripheral blood was collected from healthy laboratory members involved in this study (free of infectious diseases, without recent alcohol or nicotine consume; informed consent was signed following the consultation of the local ethics committee) into 10 ml i-PRF plain vacuum tubes (i-PRF tubes, Process for PRF, Nice, France) and immediately centrifuged at 700 r/min for 3 min at room temperature using the Duo centrifuge (Duo Zentrifuge; Mectron, Cologne, Germany). The upper liquid layer was collected being i-PRF.[33]

HOB at passage five were seeded in a density of 5×10^4 cells per well. Immediately after the seeding,

100 mg of ABSM and 100 mg of XBSM were incubated with 150 µl of i-PRF to each sample and added to the HOB. HOB with each of the two BSM without i-PRF served as the control groups. Compositions were incubated 3, 7 and 10 days at 37°C in a constant, humidified atmosphere with 95% room air and 5% CO_2. A minimum of three replications of each test was carried out.

Cell viability

To visualize cell viability, cells were stained after 3, 7 and 10 days with CellTracker (Life Technologies, Thermo Fisher Scientific, Darmstadt, Germany; catalog number: C34552). Red dye was prepared according to the manufacturer's instructions. Culture media was removed, warmed Red dye was added and plates were incubated for 30 min (37°C). After removal of the dye, serum-free medium was added and incubated for 30 min (37°C). Red fluorescence was detected with a fluorescence BZ-9000 microscope (Keyence, Osaka, Japan). Quantification of cells was conducted by ImageJ software (ACTREC, Navi Mumbai, India) as previously described.[38] In brief, images (magnification 10×) were converted to grayscale. The correction of background was conducted through image subtraction. Cell structures were extracted from the background by automatic thresholding and the area fraction (%) was calculated. Measures were made in triplication per sample (n = 3 for each group and each time point, three time points).

Cell migration

Cell attachment was studied by scratch test (ST). In brief, a scratch was manually applied to the bottom surface of the wells with a p200 pipet tip (Gilson, Middleton, USA) at days 3, 7, and 10 of incubation as described in the literature.[39] One day after ST, red staining with CellTracker was applied as described above. Quantification of cells migrated in the gap was conducted using ImageJ software in 10× fold magnification on digitalized images by means of percent area (%).[38] Selected area fraction of each image was customized according to the gap of their equivalent image on the first day of scratch. To compare results, all numbers were adjusted to an area of 100 square pixels. The measurements were carried out in triplication per group ($n = 3$ for each group and each time point, three time points).

Cell proliferation

Assessment of cell proliferation was provided using 3–(4, 5-Dimethylthiazol-2 - yl)-2, 5- diphenyltetrazolium bromide (MTT) assay on days 3, 7, and 10. MTT (200 µL, 2 mg/mL) was added to the wells and incubated for 4 h at 37°C. Then, culture medium was discarded, and 10 ml lysis buffer was added per well. The plates were read at 570 nm by a fluorescence microplate reader (Versamax, Molecular Devices, San Jose, CA, USA). Measures were made in triplication for each group (n = 3 for each group and each time point, three time points).

Real-time quantitative PCR

Evaluation of the real-time quantitative PCR (qRT-PCR) activity of alkaline phosphatase (AP), bone morphogenic protein 2 (BMP-2), and osteocalcin (OCN) was implemented. Total RNA was extracted after days 3, 7, and 10 using a commercial kit (Qiagen, Hilden, Germany). RNA was converted to cDNA by iScript cDNA synthesis kit (BioRad, Hercules, USA) according to manufacturers' instructions. Actin and GAPDH genes were used as an internal control for normalization. Primers in following sequence were applied: **Actin:** sense-GGAGCAATGATCTTGATCTT, antisense-CTTCCTGGGCATGGAGTCCT; **GAPDH:** sense-AAAACCCTGCCAATTA TGAT, antisense-CA GTGAGGGTCTCTCTCTTC; **AP:** sense- ACTGCA GACATTCTCAAAGC, antisense-GAGTGAGTGAG TGAGCAAGG; **BMP-2:** sense-(1)-CCTGAAACAGA GACCCACCC; antisense-(1)-TCTGGTCACGGGGA ATTTCG; **OCN:** sense-GSAAAGGTGCAGCCTTT GGT; antisense-GGCTCCCAGCCATTGATACAG. PCR was performed in CFX Connect Real-Time PCR Detection System (Bio-Rad, Germany) using SYBR Green Supermix (BioRad, Hercules, USA). Following proportions were applied: 11 µl of SYBR, 1 µl of primer sense, 1 µl of primer antisense, and 5 µl RNA-free water. The conditions of the thermal cycler were the following: first step –95°C for 3:00 min; second Step (repeated 39 times) –95°C for 10 s, then 58°C for 30 s and finally 72°C for 20 s; final step –65°C for 0.5 s and then 95°C for 5 s. Quantification of gene expression was conducted through ΔΔCT method. The measurements were carried out in triplication per group ($n = 3$ for each group and each time point, three time points).

Statistical analyses

The numeric results of the study were interpreted in mean values with the estimate of its standard error of the mean (SEM) and rounded to second decimal place. Data was checked on normal distribution applying Shapiro-Wilk test (SWT). In case of normally distributed values, two groups were compared via two-sided Student's *t*-test for paired samples (*t*-test). In case of non-normal distributions, Mann–Whitney test (MWT) was used to compare two groups. In order to compare

all groups, Kruskal–Wallis rank sum test (KWT) was applied. A $p \leq 0.05$ was considered to be statistically descriptive significant. Bar charts with error bars were used for data illustration.

Results

Cell viability

At day 3, ABSM-i-PRF and ABSM showed the highest cell number (48.79 ± 1.56 and 15.96 ± 9.21, respectively) and the cell number on ABSM-i-PRF was distinctively higher when compared to both XBSM groups ($p \leq 0.001$; Figure 1). In ABSM, significantly more viable cells were counted when compared to XBSM ($p \leq 0.05$) and XBSM-i-PRF had significantly higher numbers when compared to XBSM ($p \leq 0.05$). On days 7 and 10, a distinctively increased HOB growth could be observed in BSM-i-PRF- versus non-i-PRF BSM-groups ($p \leq 0.05$). However, ABSM-i-PRF remained higher than XBSM-i-PRF at both time points ($p > 0.05$) as well as ABSM remained higher than XBSM ($p \leq 0.05$). The values of ABSM-i-PRF were higher than those of XBSM during the whole period ($p \leq 0.001$). In total, there was a significant difference among ABSM-i-PRF and XBSM groups on days 7 and 10 ($p \leq 0.05$) (Figures 1 and 2; Table 1).

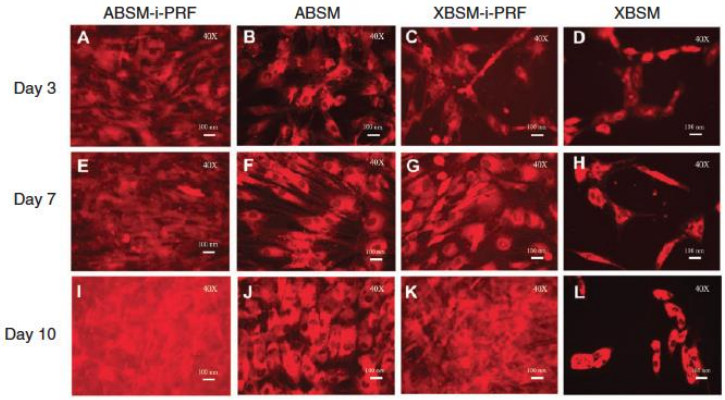

Figure 1. Exemplary figures of HOB viability (40×): ABSM-i-PRF (a, e, i), ABSM (b, f, j) as well as XBSM-i-PRF (c, g, k) and XBSM (d, h, l) on days 3, 7, and 10.

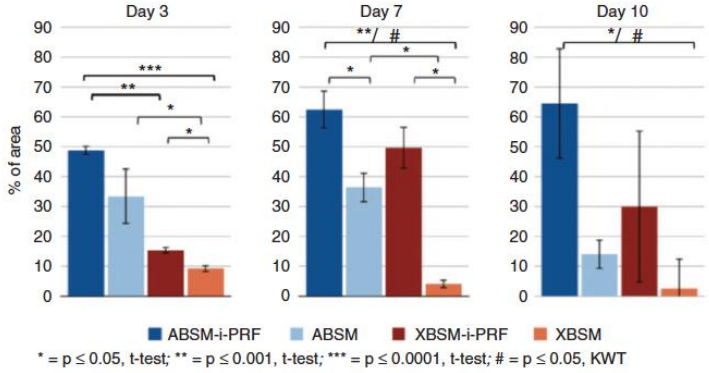

Figure 2. Bar charts: percent of viable HOB in comparison to the total area (software ImageJ (ACTREC, Navi Mumbai, India)); normal distributed variables of groups (ABSM-i-PRF, ABSM, XBSM-i-PRF, XBSM); mean values; *error bars* show SEM. *n = 3 for each group and each time point, three time points.*

Table 1. Percentage of viable HOB in relation to the whole area; statistical significance by *t*-test when comparing the groups (ABSM-i-PRF, ABSM, XBSM-i-PRF, XBSM (normal distribution)) on days 3, 7, and 10; $n = 3$ for each group and each time point, three time points.

	Cell viability by area fraction (%)					
	Day 3		Day 7		Day 10	
	ABSM-i-PRF	ABSM	ABSM-i-PRF	ABSM	ABSM-i-PRF	ABSM
Mean Value with SEM	48.79 ± 1.56	15.96 ± 9.21	62.43 ± 6.39	36.39 ± 4.93	64.48 ± 18.53	14.01 ± 4.92
Median	47.63	41.49	68.81	33	80.73	14.56
	XBSM-i-PRF	XBSM	XBSM-i-PRF	XBSM	XBSM-i-PRF	XBSM
Mean Value with SEM	15.29 ± 1.15	9.21 ± 1.11	49.67 ± 7	4.04 ± 1.23	29.97 ± 25.4	2.54 ± 1.38
Median	15.06	8.71	48.49	4.63	7.08	2.71
	P-value					
	Day 3		Day 7		Day 10	
	XBSM	ABSM-i-PRF	XBSM	ABSM-i-PRF	XBSM	ABSM-i-PRF
XBSM-i-PRF	$p \leq 0.05$[a]	$p \leq 0.001$[a]	$p \leq 0.05$[a]	$p > 0.05$	$p > 0.05$	$p > 0.05$
ABSM	$p \leq 0.05$[a]	$p > 0.05$	$p \leq 0.05$[a]	$p \leq 0.05$[a]	$p > 0.05$	$p > 0.05$
XBSM		$p \leq 0.0001$[a]		$p \leq 0.001$[a]		$p \leq 0.05$[a,b]
				$p \leq 0.05$[b]		

ABSM: allogenic bone substitute material; XBSM: xenogenic bone substitute material; OCN: osteonectin; PRF: platelet-rich fibrin; ABSM-i-PRF: allogenic bone substitute material with injectable platelet-rich fibrin; XBSM-i-PRF: xenogenic bone substitute material with injectable platelet-rich fibrin.
[a]*t*-test.
[b]Kruskal–Wallis rank sum test.

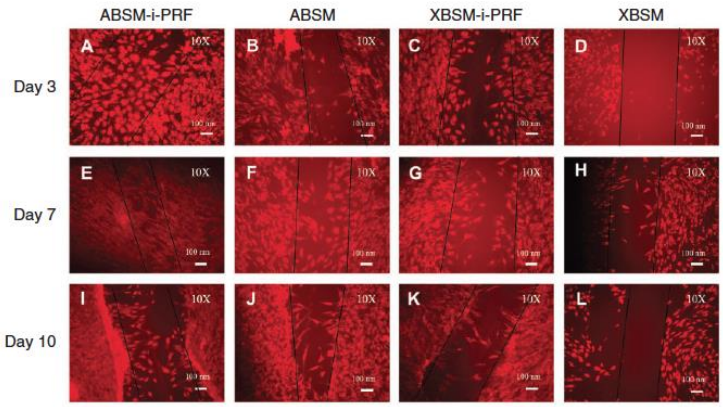

Figure 3. Exemplary figure of HOB attachment (fluorescence BZ-9000 microscope (Keyence, Osaka, Japan), enlargement 10×): groups with ABSM-i-PRF (a, e, i); ABSM (b, f, j); XBSM-i-PRF (c, g, k), and XBSM (d, h, l) at days 3, 7, and 10 after scratch test.

Cell migration

Concerning ST, extensive differences between the groups were detected. At day 3, closure of the gap was observed in all ABSM-i-PRF samples whereas no closure could be observed in the other groups (Figure 3). In accordance, the percentage of HOB in the gap of ABSM-i-PRF was almost doubled when compared to ABSM, XBSM-i-PRF and even more when compared to XBSM during the whole period ($p > 0.05$). At day 10, XBSM-i-PRF showed significantly higher values than XBSM ($p \leq 0.05$). When compared to XBSM, ABSM also showed improved

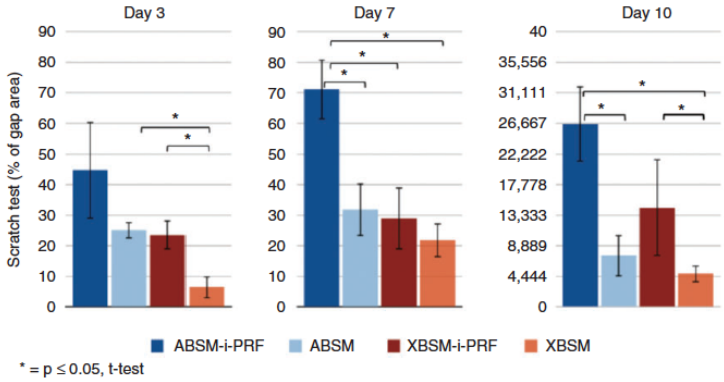

Figure 4. Bar charts: percent of HOB within the gap in comparison to the total scratch area (software ImageJ (ACTREC, Navi Mumbai, India)): ABSM-i-PRF, ABSM, XBSM-i-PRF, XBSM) on days 3, 7, and 10; normal distribution. Mean values; *error bars* show SEM. $n = 3$ for each group and each time point, three time points.

Table 2. Percentage of newly attached HOB in relation to the scratch area; statistical significance by t-test: ABSM-i-PRF, ABSM, XBSM-i-PRF, XBSM (normal distribution) on the day 3, 7, and 10.

	Scratch test (area fraction), %					
	Day 3		Day 7		Day 10	
	ABSM-i-PRF	ABSM	ABSM-i-PRF	ABSM	ABSM-i-PRF	ABSM
Mean Value with SEM	44.72 ± 15.84	25.09 ± 2.67	71.08 ± 9.76	31.84 ± 8.58	26.59 ± 5.49	7.46 ± 3
Median	33.29	25.7	67.39	40.35	30.96	6.6
	XBSM-i-PRF	XBSM	XBSM-i-PRF	XBSM	XBSM-i-PRF	XBSM
Mean Value with SEM	23.55 ± 4.76	6.45 ± 3.67	29.01 ± 10.14	21.82 ± 5.48	14.44 ± 7.03	4.82 ± 1.22
Median	26.74	4.65	22.86	23.53	8.12	4.75
	P-value					
	Day 3		Day 7		Day 10	
	XBSM	ABSM-i-PRF	XBSM	ABSM-i-PRF	XBSM	ABSM-i-PRF
XBSM-i-PRF	**$p \leq 0.05$**[a]	$p > 0.05$[a]	$p > 0.05$[a]	**$p \leq 0.05$**[a]	**$p \leq 0.05$**[a]	$p > 0.05$[a]
ABSM	**$p \leq 0.05$**[a]	$p > 0.05$[a]	$p > 0.05$[a]	**$p \leq 0.05$**[a]	$p > 0.05$[a]	**$p \leq 0.05$**[a]
XBSM		$p > 0.05$[a]		**$p \leq 0.05$**[a]		**$p \leq 0.05$**[a]

$n = 3$ for each group and each time point, three time points. (ABSM: allogenic bone substitute material; XBSM: xenogenic bone substitute material; OCN: osteonectin; PRF: platelet-rich fibrin; ABSM-i-PRF: allogenic bone substitute material with injectable platelet-rich fibrin; XBSM-i-PRF: xenogenic bone substitute material with injectable platelet-rich fibrin.)
[a]t-test.

closure of the gap at all time points ($p > 0.05$). At days 1, 7 and 10, in ABSM-i-PRF were significantly more HOB within the gap when compared to XBSM ($p > 0.05$) (Figures 3 and 4; Table 2).

Cell proliferation

The significant highest proliferation rate could be observed in samples with ABSM-i-PRF when compared to the other groups during the whole period ($p \leq 0.05$). Also, at all time points, XBSM-i-PRF as well as ABSM showed higher proliferation values when compared to XBSM ($p \leq 0.05$) (Figure 5; Table 3).

Real-time quantitative PCR

Alkaline phosphatase (AP) expression. A statistically significant difference was observed at day 7 between

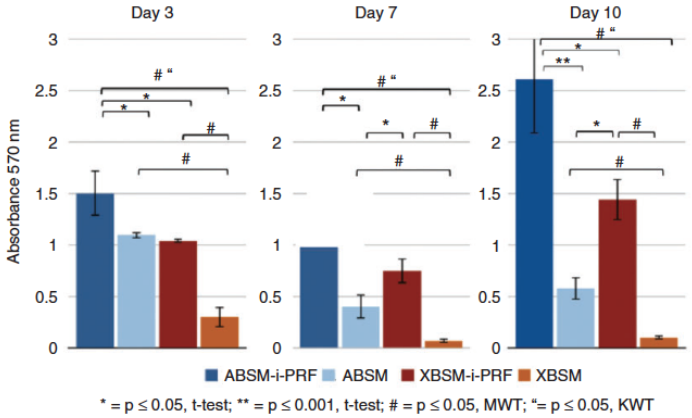

Figure 5. Bar charts showing the comparison in HOB proliferation rate (MTT) in ABSM-i-PRF, ABSM, XBSM-i-PRF with normal distribution) on days 3, 7, and 10; data of XBSM is not normally distributed; error bars show SEM. $n = 3$ for each group and each time point, three time points.

Table 3. Proliferation rate of HOB (MTT assay); statistical significance by *t*-test (ABSM-i-PRF, ABSM, XBSM-i-PRF, XBSM) on the days 3, 7, and 10.

	Proliferation rate					
	Day 3		Day 7		Day 10	
	ABSM-i-PRF	ABSM	ABSM-i-PRF	ABSM	ABSM-i-PRF	ABSM
Mean Value with SEM	1.5 ± 0.22	1.1 ± 0.03	1.08 ± 0.12	0.4 ± 0.08	2.61 ± 0.16	0.58 ± 0.1
Median	1.3	1.12	1.06	0.35	2.59	0.49
	XBSM-i-PRF	XBSM	XBSM-i-PRF	XBSM	XBSM-i-PRF	XBSM
Mean Value with SEM	1.04 ± 0.02	0.3 ± 0.1	0.75 ± 0.12	0.07 ± 0.02	1.44 ± 0.2	0.1 ± 0.02
Median	1.04	0.26	0.7	0.05	1.58	0.1

	P-value								
	Day 3			Day 7			Day 10		
	ABSM-i-PRF	XBSM-i-PRF	ABSM	ABSM-i-PRF	XBSM-i-PRF	ABSM	ABSM-i-PRF	XBSM-i-PRF	ABSM
XBSM	**$p \leq 0.05^{a,b}$**	**$p \leq 0.05^{a}$**	**$p \leq 0.05^{a}$**	**$p \leq 0.05^{a,b}$**	**$p \leq 0.05^{a}$**	**$p \leq 0.05^{a}$**	**$p \leq 0.05^{a,b}$**	**$p \leq 0.05^{a}$**	**$p \leq 0.05^{a}$**
ABSM-i-PRF		**$p \leq 0.05^{c}$**	**$p \leq 0.05^{c}$**		$p > 0.05^{c}$	**$p \leq 0.05^{c}$**		**$p \leq 0.05^{c}$**	**$p \leq 0.001^{c}$**
XBSM-i-PRF			$p > 0.05^{c}$			**$p \leq 0.05^{c}$**			**$p \leq 0.05^{c}$**

$n = 3$ for each group and each time point, three time points. (ABSM: allogenic bone substitute material; XBSM: xenogenic bone substitute material; OCN: osteonectin; PRF: platelet-rich fibrin; ABSM-i-PRF: allogenic bone substitute material with injectable platelet-rich fibrin; XBSM-i-PRF: xenogenic bone substitute material with injectable platelet-rich fibrin.)

[a] $p \leq 0.05$, Mann–Whitney test.

[b] $p \leq 0.05$, Kruskal–Wallis rank sum test.

[c] $p \leq 0.05$, *t*-test.

ABSM-i-PRF and ABSM as well as between ABSM-i-PRF/ABSM and X-BSM-i-PRF with the highest values for XBSM-i-PRF ($p \leq 0.05$) (Figure 6 (a); Table 4).

Bone morphogenetic protein 2 (BMP-2) expression. The non-significantly highest expression of BMP-2 ($p > 0.05$) was detected in groups with ABSM-i-PRF at days 3, 7, and 10 (32.9 ± 0.2, 32.87 ± 0.52, 32.42 ± 0.6,

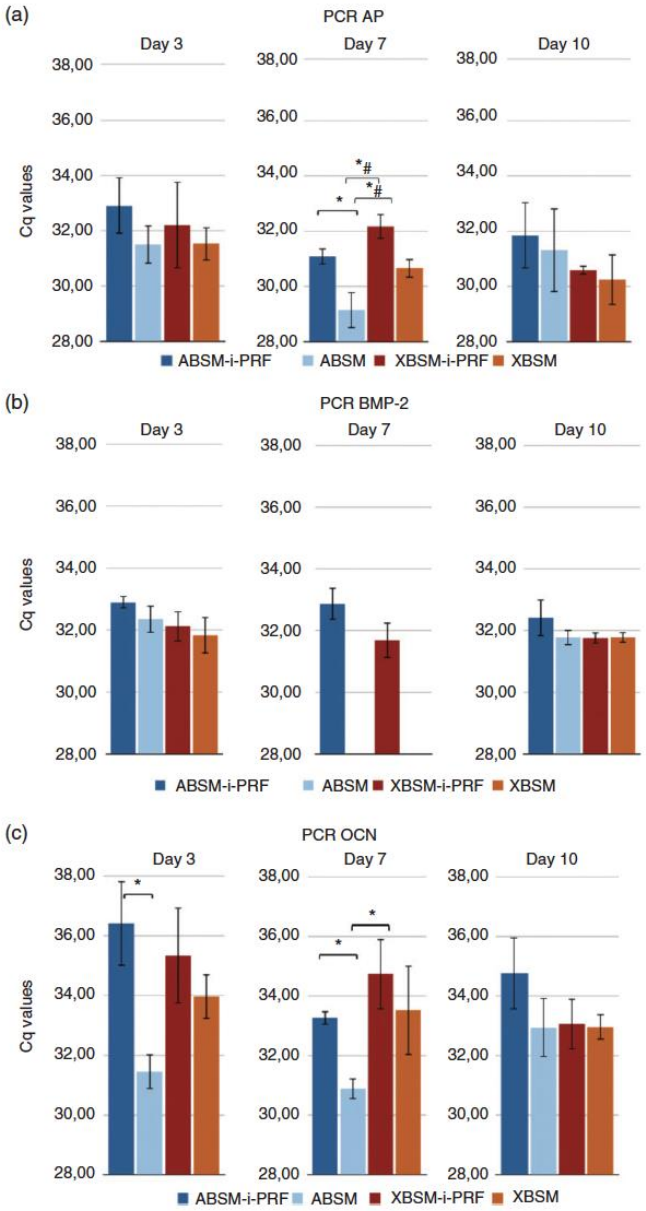

Figure 6. Bar charts showing AP (a), BMP-2 (b), and OCN expression (c) in the groups ABSM-i-PRF, ABSM, XBSM-i-PRF, XBSM on days 3, 7, and 10, measured by real-time quantitative PCR. Values of all groups were normally distributed except ABSM/XBSM in BMP expression groups; *error bars* show SEM. $n=3$ for each group and each time point, three time points. $^{*}p \leq 0.05$, *t*-test; $^{\#}p \leq 0.05$, Kruskal–Wallis rank sum test.

Table 4. Expression of AP, BMP-2, and OCN for ABSM-i-PRF, ABSM, XBSM-i-PRF, XBSM on days 3, 7, and 10.

PCR Activity of AP, BMP-2, and OCN						
AP						
	Day 3		Day 7		Day 10	
	ABSM-i-PRF	ABSM	ABSM-i-PRF	ABSM	ABSM-i-PRF	ABSM
Mean value with SEM	32.91 ± 1.02	31.50 ± 0.69	31.09 ± 0.3	29.14 ± 0.65	31.85 ± 1.2	31.31 ± 1.52
Median	32.84	31.76	31.19	29.79	32.53	32.60
	XBSM-i-PRF	XBSM	XBSM-i-PRF	XBSM	XBSM-i-PRF	XBSM
Mean value with SEM	32.21 ± 1.57	31.53 ± 0.6	32.17 ± 0.45	30.66 ± 0.34	30.59 ± 0.62	30.25 ± 0.92
Median	32.34	31.79	32.61	30.45	30.61	30.4
BMP-2						
	Day 3		Day 7		Day 10	
	ABSM-i-PRF	ABSM	ABSM-i-PRF	ABSM	ABSM-i-PRF	ABSM
Mean value with SEM	32.9 ± 0.2	32.35 ± 0.44	32.87 ± 0.52	0	32.42 ± 0.6	31.77 ± 0.25
Median	32.75	32.7	0.2	0	31.93	31.99
	XBSM-i-PRF	XBSM	XBSM-i-PRF	XBSM	XBSM-i-PRF	XBSM
Mean value with SEM	32.12 ± 0.49	31.84 ± 0.6	31.69 ± 0.57	0	31.76 ± 0.18	31.78 ± 0.17
Median	31.89	31.68	31.51	0	31.68	31.7
OCN						
	Day 3		Day 7		Day 10	
	ABSM-i-PRF	ABSM	ABSM-i-PRF	ABSM	ABSM-i-PRF	ABSM
Mean value with SEM	36.41 ± 1.4	31.45 ± 0.59	33.26 ± 0.23	30.89 ± 0.35	34.76 ± 1.1	32.94 ± 1
Median	36.79	31.71	33.07	31.24	34.68	32.26
	XBSM-i-PRF	XBSM	XBSM-i-PRF	XBSM	XBSM-i-PRF	XBSM
Mean value with SEM	35.34 ± 1.6	33.97 ± 0.74	34.73 ± 1.18	33.52 ± 1.5	33.06 ± 0.85	32.96 ± 0.43
Median	36.18	34.21	34.49	33.66	33.12	32.69
P-value						
	Day 3		Day 7		Day 10	
AP						
	ABSM	XBSM-i-PRF	ABSM	XBSM-i-PRF	ABSM	XBSM-i-PRF
ABSM-i-PRF	$p > 0.05$*	$p > 0.05$*	**$p \leq 0.05$***	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*
XBSM	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*
XBSM-i-PRF	$p > 0.05$*		**$p \leq 0.05$*†**			
BMP-2						
ABSM-i-PRF	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*
XBSM	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*
OCN						
ABSM-i-PRF	**$p \leq 0.05$***	$p > 0.05$*	**$p \leq 0.05$***	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*
XBSM	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*	$p > 0.05$*
XBSM-i-PRF	$p > 0.05$*		**$p \leq 0.05$***			

Note: Values of all groups were normally distributed except for ABSM/XBSM in BMP-2 expression groups; statistical significance by *t*-test; $n = 3$ for each group and each time point, three time points. (ABSM: allogenic bone substitute material; XBSM: xenogenic bone substitute material; OCN: osteonectin; PRF: platelet-rich fibrin.)

*$p \leq 0.05$, *t*-test.

†$p \leq 0.05$, Kruskal–Wallis rank sum test.

respectively). At day 7, in groups with non-i-PRF-BSM, there was no BMP-2 expression observed at all. With the exception of day 10, BMP-2 expression remained non-significantly higher ($p > 0.05$) in BSM-i-PRF groups when compared to their equivalent non-i-PRF-BSM groups (Figure 6(b), Table 4).

Osteonectin (OCN) expression. At days 3 and 7, the highest OCN expression of all groups could be observed in the ABSM-i-PRF group (36.41 ± 1.4 and 34.76 ± 1.1, respectively). At day 7, the highest OCN expression was shown in XBSM-i-PRF groups (34.73 ± 1.18). Significant differences were measured between the ABSM-i-PRF and ABSM groups at days 3 and 7 as well as between XBSM-i-PRF and ABSM at day 7 (all $p \leq 0.05$) (Figure 6(c), Table 4).

Discussion

In this study, a beneficial in vitro effect of i-PRF in combination with an allogenic (ABSM) and a xenogenic bone substitute material (XBSM) on HOB characteristics was evaluated. BSM in combination with i-PRF showed a significantly elevated HOB viability, attachment and proliferation as well as expression of proliferation and differentiation markers when compared to non-i-PRF-BSM. However, XBSM-i-PRF as well as XBSM showed inferiority when compared to the allogenic bone substitute (ABSM) and, to a greater extent, to ABSM-i-PRF in most of the parameters. In conclusion, the obtained data clearly demonstrate the healing-enhancement effect of the PRF when combined with BSM. Another possible approach analyzed by others may represent the use of single growth factors such as VEGF or PDGF to enhance healing.[40,41] However, due to high costs and strict regulations, the use of single growth factors in addition to BSM is not implemented up-to-date in the clinical pathway.

The main prerequisites for ideal BSM are both ease of its handling and application as well as biological characteristics close to autologous bone.[42] Considering that, allograft may be the BSM of choice, due to its unlimited availability, possibility of patient specific customization and its biocompatibility. Generally, ABSM has the potential to induce endochondral bone formation, that is feasible by incorporated BMPs and collagen type I,[43–45] although limited in comparison to autologous bone.[46] First, this might be due to storage, processing, and sterilization procedures;[47] second, because of immunologic responses of the recipient.[48] However, on the final stage of incorporation, there are no histologic differences between allogenic and autologous graft.[43,49] Thus, Soardi et al. have reported allograft to have best outcomes compared to other types of BSM in augmentation of severely atrophied alveolar ridges.[50] Tilaveridis et al. gave evidence for effectiveness of ABSM in sinus augmentation procedures in a retrospective study of critical cases.[1] It was also demonstrated that ABSM is equivalent to autogenous bone in augmentation of single tooth defects.[2] Tong et al. stated an increased effectiveness of demineralized freeze-dried bone in comparison to autologous bone considering implant survival.[51] Besides, Solakoglu et al. gave evidence for a low risk of residual alloimmunization of processed allografts.[52]

Recently, it was shown that low speed centrifugation has a direct effect on matrix contents of PRF.[32,53,54] Thus, reducing the relative centrifugation force, a new protocol of PRF processing was proposed that can produce a stable (advanced-PRF, A-PRF) or liquid (injectable, i-PRF) membrane.[32] In accordance, the main difference of i-PRF from conventional PRF is the lower speed of preparation and decreased time of centrifugation, that enables to keep some important cellular components in it.[16,32,33] Due to these changes in centrifugation time, a significantly increase in leukocyte number in the PRF matrix and an increased expression of growth factors was seen.[32,33,53,54] It was reported that i-PRF shows a significantly higher long-term release of growth factors and a higher fibroblast migration when compared to other platelet concentrates.[33] Considering that, i-PRF was chosen in the present study to test its synergic effect with different BSM.

Although i-PRF has shown success in various clinical implementation,[34,55] there is a lack of reliable data considering preclinical and clinical issues of combination of i-PRF with ABSM and XBSM.[56–58] Previous studies reported that PRF releases autologous growth factors gradually and expresses stronger and more durable effect on proliferation and differentiation of HOB in vitro more than any other platelet concentrates.[21,22] In the present study, significant differences in between the groups in terms of HOB viability, attachment and proliferation were seen. Here, ABSM-i-PRF showed the most favorable in vitro results in comparison to XBSM-i-PRF, ABSM, and XBSM. This is in accordance with an animal study by Karayürek et al. that found an increased bone healing in the group with PRF and autografts. Even if the combination of β-tricalcium-phosphate with PRF could not provide superiority in terms of bone regeneration, the immunohistochemical results showed a high expression of proliferation marker such as osteopontin and osteonectin.[59] Some studies investigated the effect of PRF on XBSM.[60] However, there is only low-level evidence of benefits of the combination of PRF with XBSM in terms of graft maturation when transferred in the clinical setting.[60–62]

To the best of our knowledge, no other study analyzed the in vitro effect of i-PRF on HOB when

combined with ABSM. However, the beneficial effect on ABSM was evaluated clinically as Choukron et al. demonstrated a reduction of healing time prior to implant placement after sinus augmentation with freeze-dried ABSM in combination with i-PRF.[21,22] Addition of PRF to ABSM has shown to enhance bone maturation and to shorten the time span before implant placement.[63] This could be explained by the (minimal) osteoinductive potential of ABSM which may be further enhanced by platelet concentrates' cellular components and could approximate induction properties of ABSM to autologous bone transplants, providing new approaches in bone regeneration. Interestingly, BMP-2 expression, despite the late HOB passage, showed relatively high expression rate in all groups except for the non-i-PRF groups on day 7 where it failed to show any expression at all.

This study suffers from some limitations. First, this is an in vitro pilot study. Therefore, results should be re-evaluated in an animal model and further prospective trials before implementation in the clinical workflow. Second, future studies should also include HOB alone with and without PRF to evaluate the effects of the biomaterials and PRF alone. Third, growth factor expression of PRF varies intra- and interindividual. Therefore, a cohort study may help to further characterize "optimal" expression levels for future clinical use.

Choukroun et al. revealed that the donor-related values of the platelet distribution and the leukocytes number by different protocols of PRF preparation were approximately similar.[33] On the other hand, there is emerging evidence of interindividual differences (age, sex, underlying conditions) concerning mainly the platelet counts of the different donors that seems to correlate with the fibrin network density and released growth factor levels.[64] Here further studies are also needed.

Conclusion

i-PRF in combination with ABSM and – to a lesser extent – to XBSM enhances proliferation, cell migration and differentiation of HOB when compared to non-i-PRF-BSM in vitro. This could ultimately result in a clinical advantage in terms of faster bony healing.

Authors' contribution

The methodology followed in this study was taken care of by PWK, SK, and SB; SK, SB, AP, PWK were involved in Formal analysis and investigation; SK, PWK were involved in writing and original draft preparation; SK, SB, AP, DT, BAl-N, PWK took part in writing, review and editing; PWK and BAl-N were involved in supervision. SK und SB contributed equally to this study.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: SK received a research grant from the Osteology Foundation. AP, BAl-N and PWK received honoraria for speaking at symposia by Straumann. BAl-N received honoraria for speaking at symposia by Straumann and Geistlich.

Ethical approval

All procedures participants were in accordance with the ethical standards of the local research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

For venepuncture and collection of peripheral blood of healthy volunteers, informed consent was obtained from all individual participants included in the study. In accordance to the local Ethical Committee, further formal approval of this kind of study was not necessary.

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1. Activation of human osteoblasts via different bovine bone substitute materials with and without injectable platelet rich fibrin in vitro
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Activation of Human Osteoblasts via Different Bovine Bone Substitute Materials With and Without Injectable Platelet Rich Fibrin *in vitro*

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Introduction: The aim of the *in vitro* study was to compare the effect of four bovine bone substitute materials (XBSM) with and without injectable platelet-reach fibrin for viability and metabolic activity of human osteoblasts (HOB) as well as expression of alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP-2), and osteonectin (OCN).

Materials and Methods: Cerabone® (CB), Bio-Oss® (BO), Creos Xenogain® (CX) and MinerOss® X (MO) ± i-PRF were incubated with HOB. At day 3, 7, and 10, cell viability and metabolic activity as well as expression of ALP, OCN, and BMP-2, was examined.

Results: For non-i-PRF groups, the highest values concerning viability were seen for CB at all time points. Pre-treatment with i-PRF increased viability in all groups with the highest values for CB-i-PRF after 3 and 7 and for CX-i-PRF after 10 days. For metabolic activity, the highest rate among non-i-PRF groups was seen for MO at day 3 and for CB at day 7 and 10. Here, i-PRF groups showed higher values than non-i-PRF groups (highest values: CB + i-PRF) at all time points. There was no difference in ALP-expression between groups. For OCN expression in non-i-PRF groups, CB showed the highest values after day 3, CX after day 7 and 10. Among i-PRF-groups, the highest values were seen for CX + i-PRF. At day 3, the highest BMP-2 expression was observed for CX. Here, for i-PRF groups, the highest increase was seen for CX + i-PRF at day 3. At day 7 and 10, there was no significant difference among groups.

Conclusion: XBSM sintered under high temperature showed increased HOB viability and metabolic activity through the whole period when compared to XBSM manufactured at lower temperatures. Overall, the combination of XBSM with i-PRF improved all cellular parameters, ALP and BMP-2 expression at earlier stages as well as OCN expression at later stages.

Keywords: bone substitute, bovine bone, platelet rich fibrin (PRF), *in vitro,* osteoblast, vitality, proliferation, PCR

INTRODUCTION

The composition of bovine bone substitutes is similar to human bone due to the preserved microstructure of the osseous frame (Glowacki, 2005; Yamada and Egusa, 2018). These xenografts are known for osteoconduction and a low (if any) absorbability rate (Klein et al., 2013b; Dau et al., 2016). Deproteinization potentially allows elimination of transmission risk and antigenicity (Lei et al., 2015). However, different cleaning and manufacturing methods may affect the regeneration capacity of the a bovine bone substitute material. For example, manufacturing of deproteinized bovine bone by sintering consists of high temperature treatment with stepwise heating up to >1,000°C leading to the removal of all organic components including collagen (Lei et al., 2015). On contrary, manufacturing with lower temperatures usually comprise an additional chemical treatment, i.e., with sodium hydroxide with efficiently inactivated viruses (Tadic and Epple, 2004). Cerabone® (Botiss, Zossen, Germany) is produced via three-stage temperature treatment including a final sintering at >1,200°C, hence all organic compounds are removed and potential prions, bacteria and viruses are eliminated. This preparation process might alter the microstructure (Perić Kačarević et al., 2018). However, it has been shown that Cerabone® resembles the structure of natural bone with high porosity and rough surface (Trajkovski et al., 2018). Bio-Oss® (Geistlich Pharma AG, Wolhusen, Switzerland) has a fiber-like surface with a much smaller crystal size (Barbeck et al., 2015; Perić Kačarević et al., 2018). It is manufactured at a lower temperature of 300°C followed by sodium hydroxide treatment (Kübler et al., 2004); thus, it is considered to be a hydroxyapatite ceramic with a high porosity including large interconnective pores and residual proteins (Kübler et al., 2004; Klein et al., 2009). Creos Xenogain® (Nobel Biocare GmbH, Gothenburg, Sweden) is produced by sodium hypochloride treatment followed by heating under 400°C. MinerOss® X (BioHorizons, Birmingham, United Kingdom) is also produced via low-heat processing of bovine bone, preserving the coarseness of bone with a high porosity.

In *in vitro* and *in vivo* studies, for injectable platelet-rich fibrin (i-PRF) a high share of leukocytes and platelets was proven. It promotes fibroblast migration and has a potential to release higher concentration of cytokines and selective growth factors over time when compared to PRP/L-PRF and A-PRF (Ghanaati et al., 2014; El Bagdadi et al., 2017; Miron et al., 2017; Wend et al., 2017; Choukroun and Ghanaati, 2018; Wang et al., 2018). Additionally, due to its consistency as well as composition, i-PRF can be used in combination with various biomaterials in order to increase their bioactivity in bone/soft tissue regeneration, and to improve healing in impaired wound healing cases (Wend et al., 2017; Miron and Zhang, 2018; Abd El Raouf et al., 2019). Thus, a combination of a bovine bone substitute material with i-PRF may be promising in terms of soft and hard tissue regeneration (Mourão et al., 2015). In our previous *in vitro* study (Kyyak et al., 2020), we compared an allograft and a bovine bone substitute material with and without i-PRF in regard of their effect on human osteoblasts' viability, gap closure and metabolic activity. Here, the allogenic material showed an improved performance, possibly due to its (minimal) osteoinductive potential. The previous study was limited as there was only one commercially available bovine bone substitute material under examination. Thus, the aim of the study was to compare four different commercially available bovine bone substitutes with and without i-PRF for viability, metabolic activity, and differentiation of human osteoblasts. The null hypothesis was that pre-treatment of bovine bone substitute materials with i-PRF affects osteoblast viability, metabolic activity, and differentiation. A secondary hypothesis was that there are also differences between the different xenogenic materials.

MATERIALS AND METHODS

Bone Substitute Materials

In the study, four bone substitute materials of bovine origin and their combination with i-PRF were included: cerabone® (CB, botiss biomaterials GmbH, Zossen, Germany, granularity: 1–2 mm), Bio-Oss® (BO, Geistlich Pharma AG, Wolhusen, Switzerland, granularity: 1–2 mm), CREOS Xenogain® (CX, Nobel Biocare GmbH, Gothenburg, Sweden, granularity: 1–2 mm), MinerOss® X (MO, BioHorizons, Birmingham, United Kingdom, granularity: 0.5–1 mm).

I-PRF

In accordance with the ethical standards of the national research committee (Ärztekammer Rheinland-Pfalz, no. "2019-14705_1"), 10 ml peripheral venous blood per sample was collected from three healthy donors without severe illnesses after puncture of the cephalic or the median cubital vein. The vacutainer system and specific sterile plain vacuum tubes with additional silicone within their coating surface for solid (A-PRF+, Mectron, Carasco, Italy) and liquid PRF were used, respectively (iPRF, Mectron, Carasco, Italy). PRF was directly manufactured at a fixed angle rotor with a radius of 110 mm with 1,200 rpm and a relative centrifugal force of 177 g for 8 min (Duo centrifuge, Mectron, Carasco, Italy) after manufacturer's instructions as described (Blatt et al., 2020).

Cells

For the *in vitro* study, commercially available human osteoblasts from one donor were chosen (HOB, PromoCell, Heidelberg, Germany). A standard HOB medium was applied for cultivation including fetal calf serum (FCS, Gibco Invitrogen, Karlsruhe, Germany), Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen), dexamethasone (100 nmol/l, Serva Bioproducts, Heidelberg, Germany), L-glutamine (Gibco Invitrogen), and streptomycin (100 mg/ml, Gibco Invitrogen). Cultivation of HOB was administered at the air temperature of 37°C, 95% humidity 95 and 5% of CO_2. The passaging of HOB was carried out when reaching 70% confluence by application of 0.25% trypsin (Seromed Biochrom KG, Berlin, Germany). Passage five HOB were seeded in a density of 5×10^4 cells per well. Afterward, 100 mg of each bone substitute material were added to the corresponding wells with HOB. Into the first half of the wells, 150 µl of i-PRF was applied, one well with each bone substitute material was left without i-PRF. Incubation of the

compositions was divided into 3, 7, and 10 days at 37°C, 95% humidity, and 5% CO_2. For negative controls, wells without bone substitute material as well as with i-PRF without bone substitute material were used.

Cell Viability

For cell viability, CellTracker staining (Life Technologies, Thermo Fisher Scientific, Darmstadt, Germany; catalog number: C34552) was applied on day 3, 7, and 10. Red dye was produced following the manufacturer's instructions. After the culture media was removed, Red dye was applied into wells and incubated for 30 min in 37°C. Serum-free medium was added, following the removal of the Red dye, and incubated for 30 min in 37°C. Red fluorescence was observed using a fluorescence BZ-9000 microscope (Keyence, Osaka, Japan). ImageJ software (ACTREC, Navi Mumbai, India) was used for cell quantification (Fuchs et al., 2009). At first, images (magnification 10×) were converted to grayscale. Through image subtraction, a correction of the background was conducted. Cell structures were extracted from the background using automatic thresholding and the area fraction (%) was calculated. Measures were conducted in triplication for each group and each time point (three time points).

Cellular Metabolic Activity

Metabolic activity was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on day 3, 7, and 10. Briefly, MTT (200 µl, 2 mg/ml) was added to the wells and incubated for 4 h at 37°C. Culture medium was removed, and lysis buffer (10 ml) was pipetted into each well. Plates values were acquired using a fluorescence microplate reader (570 nm; Versamax, Molecular Devices, San Jose, CA, United States). Measures were conducted in triplication for each group and each time point (three time points).

Expression of Bone Gene Markers

In accordance with Liu et al. (2003), the runx-2-dependent early osteogenic differentiation marker collagen alkaline phosphatase (ALP) as well as the late differentiation marker osteocalcin (OCN) was examined, whose expression is also highly controlled by runx-2 levels (Stein et al., 2004). ALP plays an important regulatory role during matrix mineralization (Hessle et al., 2002; Anderson et al., 2004). For osteogenic cells, the integrin subunits β_1 and α_v have been shown to trigger effects of cytokines like bone morphogenetic protein 2 (BMP-2) (Lai and Cheng, 2005). OCN, which is only synthesized by mature osteoblasts, is directly associated with bone matrix mineralization as well (Lian et al., 1998, 2004; Klein et al., 2013a).

For this purpose, total RNA was extracted after day 3, 7, and 10 (Qiagen, Hilden, Germany). Subsequently, RNA was converted to cDNA with the help of iScript cDNA synthesis kit (BioRad, Hercules, United States) in accordance to manufacturers' recommendations. For normalization, internal control Actin and GAPDH genes were used. The sequences of the primers are presented in **Table 1**. PCR was conducted using a CFX Connect Real-Time PCR Detection System (Bio-Rad, Germany) and SYBR Green Supermix (BioRad, Hercules, United States). Following proportions were applied: 11 µl of SYBR, 1 µl of primer sense, 1 µl of primer antisense, and 5 µl RNA-free water. The conditions of the thermal cycler were the following: first step—95°C for 3 min; second step (repeated 39 times)—95°C for 10 s, then 58°C for 30 s and finally 72°C for 20 s; final step—65°C for 0.5 s and then 95°C for 5 s. Quantification of gene expression was conducted through Ct value. Measures were conducted in triplication for each group and each time point (three time points).

TABLE 1 | Primers Actin, GAPDH, ALP, OCN, and BMP-2 and their sequences.

Primers	Sequences
Actin	sense-GGAGCAATGATCTTGATCTT
	antisense-CTTCCTGGGCATGGAGTCCT
GAPDH	sense-AAAACCCTGCCAATTATGAT
	antisense-CAGTGAGGGTCTCTCTCTTC
ALP	sense- ACTGCAGACATTCTCAAAGC
	antisense-GAGTGAGTGAGTGAGCAAGG
OCN	sense-GSAAAGGTGCAGCCTTTGGT
	antisense-GGCTCCCAGCCATTGATACAG
BMP-2	sense-(1)-CCTGAAACAGAGACCCACCC
	antisense-(1)-TCTGGTCACGGGGAATTTCG

Statistical Analyses

Data was converted into mean values with the estimate of its standard error of the mean (SEM) (for parametric data) and median values (for non-parametric data). Numbers were round off to second decimal place. Normal distribution was determined by Shapiro-Wilk test (SWT). For comparison of two groups, two-sided Student's *t*-tests for paired samples (*t*-test) were applied in case of normal distributions. In case of non-normal distributions, Mann-Whitney test (MWT) was applied to compare two groups. Kruskal-Wallis rank sum test (KWT) was applied to compare all groups. A *p*-value of ≤ 0.05 was considered to be statistically descriptive significant. Data was visualized using bar charts with error bars.

RESULTS

Cell Viability

At day 3, the highest cell viability was seen in the Cerabone® (CB) group, which was significantly higher when compared to BioOss® (BO; $p \leq 0.05$, *t*-test) that showed the lowest indexes among all tested materials. Viability of Xenogain® (CX) was significantly higher when compared to controls ($p \leq 0.05$, *t*-test) (**Figures 1**, **2A**). At day 7, controls had the highest cell viability ($p > 0.05$, MWT). Additionally, when compared to other bovine bone substitute material (XBSM) groups, CB presented the highest indexes ($p > 0.05$, *t*-test), followed by CX ($p > 0.05$, *t*-test) and MO ($p > 0.05$, *t*-test). At day 10, the highest viability value was seen for CB, followed by CX (when compared to controls

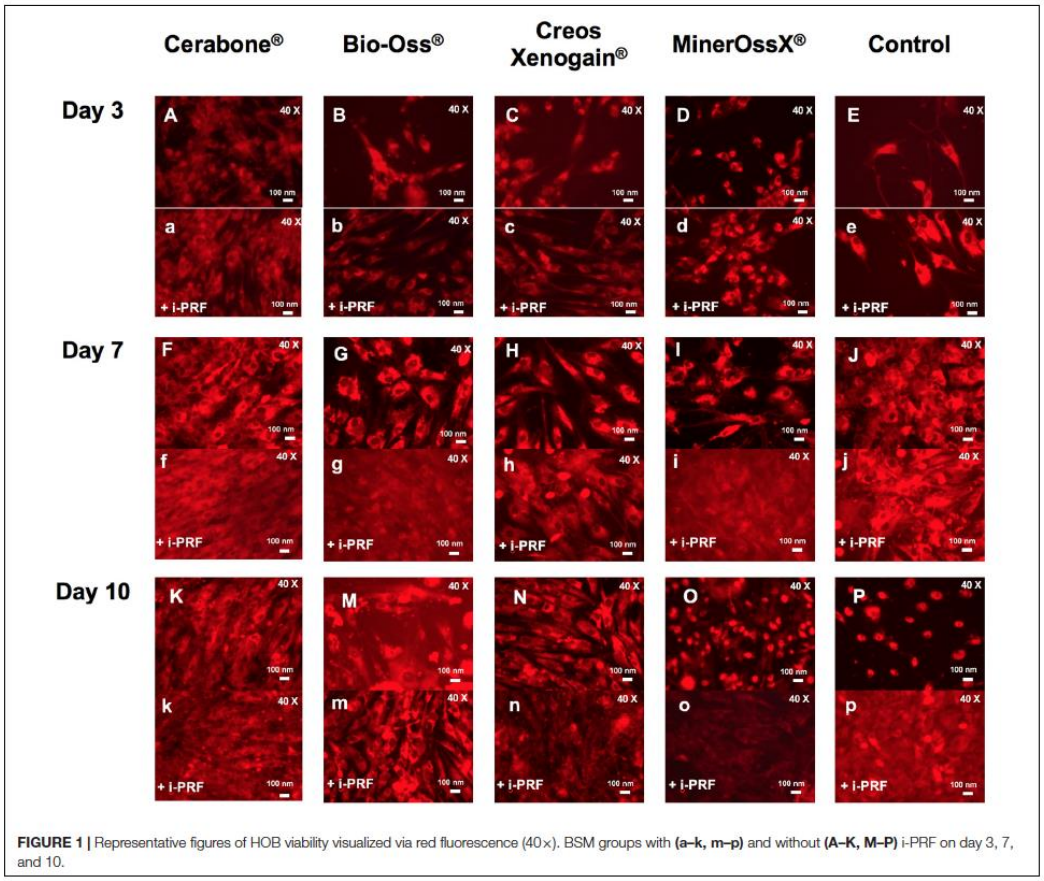

FIGURE 1 | Representative figures of HOB viability visualized via red fluorescence (40×). BSM groups with **(a–k, m–p)** and without **(A–K, M–P)** i-PRF on day 3, 7, and 10.

($p \leq 0.05$, *t*-test), BO and MO ($p > 0.05$, MWT). The lowest rate showed BO ($p > 0.05$, MWT) (**Figures 1**, **2A** and **Table 2A**).

At day 3, among i-PRF containing groups, significant higher values were observed for CB + i-PRF when compared to BO + i-PRF ($p \leq 0.001$, *t*-test), CX + i-PRF ($p \leq 0.001$, *t*-test), MO + i-PRF ($p \leq 0.05$, *t*-test), and Control + i-PRF groups ($p > 0.05$, MWT). Control + i-PRF showed the lowest viability among i-PRF-groups (significant in comparison to all others, $p > 0.05$, MWT) and among i-PRF-XBSM groups, the lowest indexes were seen for BO + i-PRF ($p > 0.05$, *t*-test) (**Figures 1**, **2** and **Table 2**). At day 7, the highest increase in viability was observed in CB + i-PRF groups [when compared to BO + i-PRF ($p \leq 0.05$, MWT), CX + i-PRF ($p \leq 0.05$, *t*-test), controls ($p > 0.05$, *t*-test)] and MO + i-PRF groups ($p > 0.05$, MWT). At day 10, the highest viability was seen for controls, followed by CX + i-PRF [when compared to BO + i-PRF ($p \leq 0.05$, *t*-test), CB + i-PRF, and MO + i-PRF (each: $p > 0.05$, MWT)] (**Figures 1**, **2B** and **Table 2B**).

All i-PRF treated groups showed higher values in comparison to their equivalent pure non-i-PRF groups through the whole period. At day 7, values of all groups doubled in comparison to day 3 and were the highest of all the periods (**Figures 1**, **2** and **Table 2**).

Cell Metabolic Activity

On the third day, MTT test showed the non-significant highest metabolic activity in MO ($p > 0.05$, *t*-test), followed by CX ($p > 0.05$, *t*-test). The least metabolic activity was observed for BO ($p > 0.05$, *t*-test). At day 7, the non-significant highest values were observed for CB, followed by controls ($p > 0.05$, *t*-test, MWT). At day 10, considerably higher values were seen in CB [when compared to BO ($p > 0.05$, MWT), CX ($p \leq 0.01$, *t*-test), MO ($p \leq 0.05$, *t*-test), and controls ($p > 0.05$, *t*-test)], followed by controls (in comparison to MO ($p \leq 0.05$, *t*-test), CX ($p > 0.05$, *t*-test), and BO ($p > 0.05$, MWT) (**Figure 3A** and **Table 3A**). At day 3, 7, and 10 among i-PRF-groups, the non-significant highest

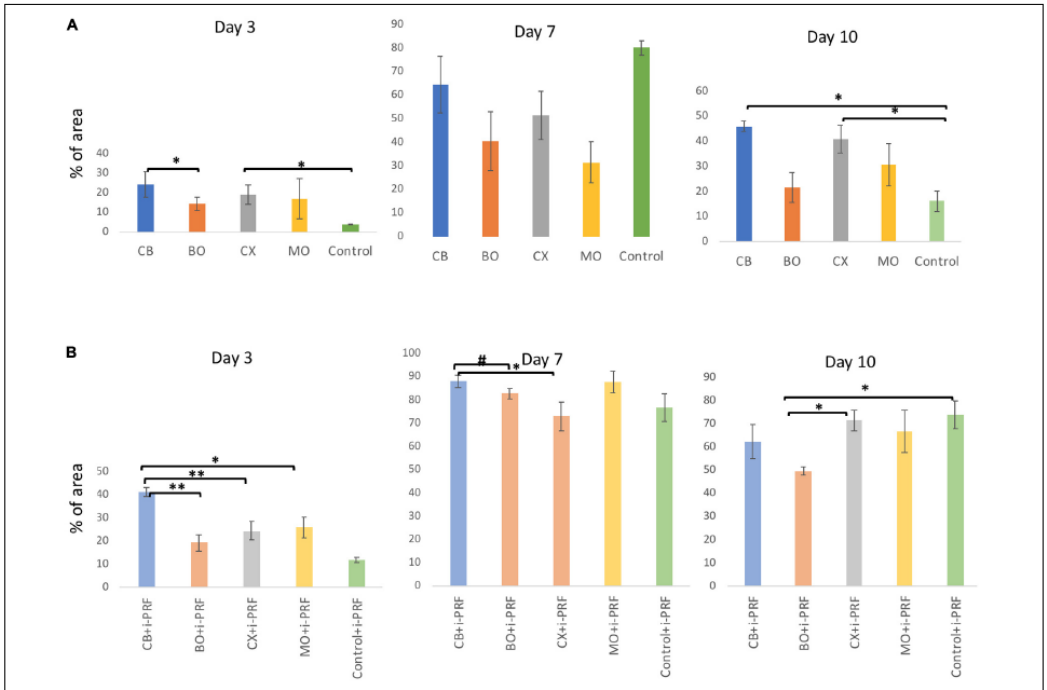

FIGURE 2 | Bar charts: viable HOB (%) with XBSM **(A)** and XBSM with i-PRF **(B)** on day 3, 7, and 10 (area fraction, ImageJ). mean values; error bars show SEM; in triplication for each group and each time point, three time points. $^{*}p \leq 0.05$, *t*-test; $^{**}p \leq 0.001$, *t*-test; $^{\#}p \leq 0.05$, MWT.

metabolic activity was seen in CB + i-PRF ($p > 0.05$, *t*-test) and controls + i-PRF ($p > 0.05$, *t*-test). The lowest metabolic activity was seen for MO + i-PRF on day 3 and 7 as well as BO + i-PRF on day 10 (**Figure 3B** and **Table 3B**).

Overall, groups containing i-PRF showed a higher value than non-i-PRF groups, except for MO on day 3. Metabolic activity levels of all groups had a general tendency to decline through the whole period (**Figure 3** and **Table 3**).

Expression of Bone Gene Markers

Alkaline Phosphatase (ALP) Expression

On day 3, 7, and 10, all groups showed almost the same ALP expression level ($p > 0.05$, *t*-test), except MO, which didn't show any expression at all through the whole period. On day 3 and 10, in the i-PRF-groups, the values were also almost on the same level ($p > 0.05$, *t*-test) with no expression in MO + i-PRF on day 10. At day 7, the highest expression was observed in BO + i-PRF [when compared to MO + i-PRF ($p \leq 0.05$, *t*-test)], followed by CB + i-PRF ($p > 0.05$, *t*-test).

Overall, on day 3, 7, and 10, i-PRF-groups had slightly increased ALP expression over non-treated bone substitute materials, with the exception of CX + i-PRF on day 7 and MO + i-PRF on day 10.

Osteonectin (OCN) Expression

On day 3, CB had the highest OCN expression [significant in comparison to BO ($p \leq 0.05$, *t*-test)]. On day 7, CX showed a significantly increased expression when compared to BO ($p \leq 0.05$, *t*-test) and (non-significant) to CB ($p > 0.05$, *t*-test). On day 10, CX had significant highest values when compared to BO ($p \leq 0.05$, *t*-test) and CB ($p \leq 0.001$, *t*-test). There was no expression in MO through the whole period. On day 3, among i-PRF treated groups, the non-significant highest value of all groups was observed in CX + i-PRF ($p > 0.05$, *t*-test), the non-significant lowest in CB + i-PRF ($p > 0.05$, *t*-test). MO + i-PRF showed no OCN expression. On day 7, values of CX + i-PRF were higher than of MO + i-PRF ($p \leq 0.05$, *t*-test) and CB + i-PRF ($p > 0.05$, *t*-test), followed by BO + i-PRF ($p > 0.05$, *t*-test). On day 10, CX + i-PRF showed the non-significant highest expression among treated groups ($p > 0.05$, *t*-test). Through the whole period, pre-treated groups showed increased expression rates when compared to non-treated XBSM, except for MO + i-PRF on day 3.

Bone Morphogenetic Protein 2 (BMP-2) Expression

Considering BMP-2 expression, on the day 3, the significant highest rate was observed in CX in comparison to BO ($p \leq 0.05$,

TABLE 2 | Cell viability of XBSM **(A)** and XBSM with i-PRF **(B)** by area fraction (%) on days 3, 7, and 10; experiments in triplication for each group and each time point, three time points.

A

XBSM	Day3					Day 7					Day 10				
	CB	BO	CX	MO	Control	CB	BO	CX	MO	Control	CB	BO	CX	MO	Control
*Mean value with SEM/**Median	*24.27 + 6.63	*14.37 + 3.29	*19.11 + 4.81	*17.10 + 10.27	*3.75 ± 0.28	*64.46 + 11.99	**28.50	*51.51 + 10.21	**28.00	**80.53	*46.0 + 2.14	**24.77	*40.82 + 5.6	**30.42	*16.19 + 4.18

*Mean values for parametric data.
**Median values for non-parametric data.

B

XBSM	Day3					Day 7					Day 10				
	CB+i-PRF	BO+i-PRF	CX+i-PRF	MO+i-PRF	Control+i-PRF	CB+i-PRF	BO+i-PRF	CX+i-PRF	MO+i-PRF	Control+i-PRF	CB+i-PRF	BO+i-PRF	CX+i-PRF	MO+i-PRF	Control+i-PRF
*Mean value with SEM/**Median	*41.25 + 1.92	*19.33 + 3.68	*24.49 ± 4.07	*25.95 + 4.51	**11.2	*87.98 + 2.44	**84.34	*73.08 + 6.11	**90.19	*76.7 + 5.86	**56.22	*49.71 + 1.86	*71.72 + 4.44	**64.42	*73.95 + 6.15

*Mean values for parametric data.
**Median values for non-parametric data.

t-test), followed by CB ($p > 0.05$, *t*-test). On day 7 and 10, there was no considerable difference among the groups. Through the whole period, there was no expression in MO. On day 3, among the i-PRF-groups, CX + i-PRF demonstrated a significant increase when compared to MO + i-PRF ($p \leq 0.05$, *t*-test) as well as a non-significant increase when compared to BO + i-PRF and CB + i-PRF (both $p > 0.05$, *t*-test). On day 7 and 10, there was no considerable difference among the groups with the exception of no expression of MO + i-PRF on day 10.

To sum up, on day 3, there was increased BMP-2 expression in all i-PRF-groups when compared to non-i-PRF-XBSM. On day 7, a positive effect of adding i-PRF on BMP-2 expression was seen in BO + i-PRF and MO + i-PRF, and on day 10 in BO + i-PRF.

DISCUSSION

For this *in vitro* study, four commercially available xenogenic, bovine bone substitute materials (XBSM)—alone and in combination with injectable platelet-rich fibrin (i-PRF) were evaluated regarding their biological effect on human osteoblast cells (HOB) after 3, 7 and 10 days. Cell viability, metabolic activity as well as expression of three bone regeneration markers (alkaline phosphatase (ALP), osteonectin (OCN), and bone morphogenic protein-2 (BMP-2) were analyzed. As a result, especially the high-sintered group showed beneficial *in vitro* effects when compared to low-sintered XBSM. Besides, addition of i-PRF to XBSM resulted in a significantly increased biological activity of HOB in most of the cases.

The main difference among the four XBSM is the preparation process, namely the temperature. They have a hydroxyapatite phase (Bohner, 2000), which causes a good biocompatibility due to similarity with crystalline phase of human bone, high porosity and micro-architecture (Laschke et al., 2007). Okumura et al. gave evidence that the reason of early osteogenesis on hydroxyapatite lies in the faster initial attachment of HOB (Stephan et al., 1999; Okumura et al., 2001). It was also reported that bovine hydroxyapatite materials treated at different temperatures show significant variation in osteoblastic activity because of changed surface roughness and biological performance (osteoconductivity) (Ong et al., 1998; Perić Kačarević et al., 2018; De Carvalho et al., 2019), which may result in different healing outcomes (Barbeck et al., 2015; Perić Kačarević et al., 2018). In our study, XBSM sintered at high temperatures [cerabone® (CB)] showed a significantly increased HOB viability and metabolic activity when compared to other materials processed at lower temperatures. CB is composed of hydroxyapatite with traces of calcium oxide with a porous bone-like morphology (Tadic and Epple, 2004). Being sintered at a high temperature (>1,200°C), it loses all organic compounds. It was reported, that CB presents the highest level of hydrophilicity in comparison to Bio-Oss® (BO) (Trajkovski et al., 2018). Besides, in 1,200°C sintered bovine hydroxyapatite, additional traces of NaCaPO4 and CaO were detected, which could result from decomposition of the bone carbonate and could improve HOB reaction (Tadic and Epple, 2004) as detected in the present study.

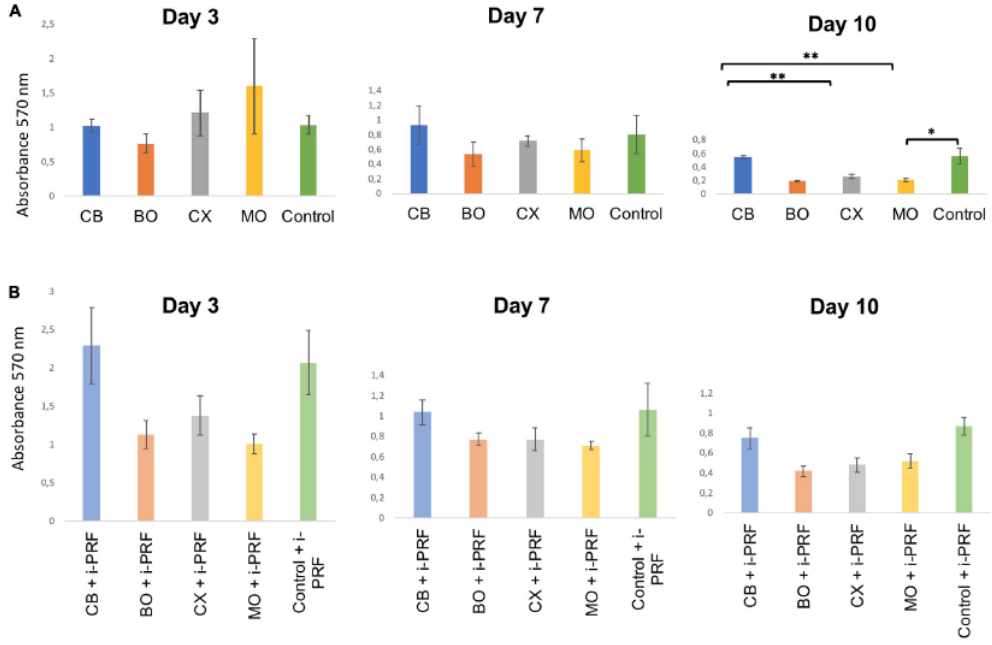

FIGURE 3 | Bar charts: HOB metabolic activity (MTT) in groups with XBSM **(A)** and XBSM with i-PRF **(B)** on day 3, 7, and 10; mean values; error bars show SEM; in triplication for each group and each time point, three time points. *$p \leq 0.05$, *t*-test; **$p \leq 0.001$, *t*-test.

Additionally, when considering the carbonate component, the influence of the surface energy of the bone substitute material may also increase initial HOB attachment and proliferation. Thus, the strengthening of the polar components of the dense surface of a bone substitute material may enhance HOB attachment and osteoconduction (Redey et al., 2000). The temperature of processing effects the elimination of carbonate content in the bone, which can be only initiated at 400°C and higher (Tadic and Epple, 2004). Besides, the high sintering temperature increases crystallinity, subsequently lowers biodegradation rates and increases volume stability (Ong et al., 1998; Bohner, 2000; Accorsi-Mendonça et al., 2008; Kusrini and Sontang, 2012; Riachi et al., 2012). On the other hand, it was stated in another study, that high-temperature sintering of a XBSM did not affect phase stability, densification behavior, fluid intrusion, and porosity when compared to non-sintered XBSM (Gehrke et al., 2019). In addition, no clinical long-term influence of osseous healing using differently processed bone substitute materials was found. Though, Kapogianni et al. (2019) analyzed samples from biopsies 6 months after sinus floor evaluation and after this considerable amount of time in a biological less demanding defect, no differences can be expected (Rickert et al., 2012; Kapogianni et al., 2019).

Despite the claim of no organic component, histological analyzes gave evidence for (xenogenic) organic remnants in XBSM treated under lower temperature, which may lead to decreased biocompatibility and osteoconductivity (Piattelli et al., 1999). BO is a carbonated hydroxyapatite, containing water, with porous granulate morphology and nanocristallinity (Tadic and Epple, 2004). It is manufactured at a temperature of 300°C, thus, is considered to include residual proteins (Kübler et al., 2004). In the present *in vitro* investigation, BO showed less distinct results for cell viability and metabolic activity as compared to other XBSMs. These findings are in accordance with other *in vitro* studies (Kübler et al., 2004; Liu et al., 2011). Sufficient osteogenic cell adhesion a bone substitute material is important for cellular proliferation, differentiation and matrix synthesis. Whereas initial cell attachment is based on unspecific cell-substrate interactions, later cell adhesion displays complex interactions between extracellular ligands and specific cellular receptors with high impact on further intracellular signal transduction (Keselowsky et al., 2007). Integrin receptors are transmembrane heterodimers consisting of non-covalently associated α and β sub-units. The sub-units β_1 and α_v have affinity to extracellular matrix proteins like fibronectin, collagen, and osteonectin via the RGD tri-peptide sequence (Heller et al., 2018). Integrin-mediated outside-in-signaling has

TABLE 3 | HOB metabolic activity (MTT, absorbance 570 nm) in groups with XBSM **(A)** and XBSM with i-PRF **(B)** on days 3, 7, and 10; in triplication for each group and each time point, three time points.

A

XBSM	Day 3					Day 7					Day 10				
	CB	BO	CX	MO	Control	CB	BO	CX	MO	Control	CB	BO	CX	MO	Control
*Mean value with EM/**Median	*1.02 ± 0.09	*0.77 ± 0.14	*1.21 ± 0.33	*1.6 ± 0.69	*1.04 ± 0.14	**1.19	*0.54 ± 0.16	*0.72 + 0.07	*0.59 ± 0.16	**0.76	*0.55 ± 0.02	**0.2	*0.26 ± 0.03	*0.21 ± 0.03	*0.56 ± 0.11

*Mean values for parametric data.
**Median values for non-parametric data.

B

XBSM	Day3					Day 7					Day 10				
	CB+i-PRF	BO+i-PRF	CX+i-PRF	MO+i-PRF	Control+i-PRF	CB+i-PRF	BO+i-PRF	CX+i-PRF	MO+i-PRF	Control+i-PRF	CB+i-PRF	BO+i-PRF	CX+i-PRF	MO+i-PRF	Control+i-PRF
*Mean value with SEM/**Median	*2.29 ± 0.5	*1.13 ± 0.18	*1.38 + 0.26	*1.01 ± 0.14	*2.07 + 0.42	*1.04 ± 0.12	*0.77 ± 0.06	*0.77 ± 0.11	*0.71 ± 0.04	*1.06 ± 0.26	*0.75 + 0.1	*0.42 ± 0.05	*0.48 ± 0.07	*0.52 ± 0.07	*0.87 ± 0.09

*Mean values for parametric data.
**Median values for non-parametric data.

been shown to regulate osteogenic cytoskeleton organization and gene expression (Anselme, 2000; El-Ghannam et al., 2004). Furthermore, during osteoblast/substrate interactions, the expression of these adhesion molecules is modified according to distinct surface characteristics (Anselme, 2000; Klein et al., 2013a). In the present study, ALP, OCN (early as well as late osteogenic differentiation markers), and BMP-2 expression of BO without and with i-PRF was comparably high. In combination with i-PRF, Creos Xenogain® (CX) showed a significantly elevated OCN expression through the whole period in comparison to other groups. However, the results of gene expression markers were inconsistent and it is possible that the inclusion of other gene expression markers such as type I collagen, Runt-related transcription factor 2 (Runx2) and Osteopontin would have shown different results.

Cell viability/metabolic activity of CX and MO more or less correlated to each other. MO is produced via low-heat processing of bovine bone, preserving the coarseness of bone with its high porosity. In a recent preclinical *in vivo* study, MO showed more osteogenic cells as well as more newly formed bone when compared to BO and autogenous bone (Esfahanizadeh et al., 2019). Nevertheless, the impact if cells are seeded on the well and the BSM is added afterward (Khanijou et al., 2020) or if the cells have been seeded on the BSM itself (Parisi et al., 2020) remains unclear.

Interactions of biomaterials such as BSM with the surrounding microenvironment define the respective biocompatibility and biochemical signaling pathways might play key roles in determining the materials' success after implantation (Rahmati et al., 2020). *In vitro* assessment of cell metabolic activity may allow conclusions to be drawn about biocompatibility of biomaterials, and cells that are metabolically active are a precondition for osteoconductivity and osteoinductivity. But it should be clearly understood that *in vitro* studies still display only a limited part of the general *in vivo* set-up and there might be a substantial gap between cellular biocompatibility and *in vivo* models (Reichert et al., 2009; Rahmati et al., 2020). For example, surface characteristics of hydroxyapatite changes after getting in contact with blood proteins and extracellular matrix components (Herten et al., 2009). Thus, monotonous conditions of *in vitro* studies may distort *in vivo* results.

To the best of our knowledge, there are no *in vitro* studies on combination of i-PRF with different XBSMs. In a clinical study, Zhang et al. (2012) showed improvement in parameters of bone regeneration when adding PRF to XBSM but there was no statistical significance. In our previous *in vitro* study, we revealed that allogenic bone substitute material with i-PRF has a significant higher impact on HOB viability, migration and metabolic activity when compared to BO with i-PRF. Still, i-PRF-BO showed better results when compared to non-i-PRF-BO groups (Kyyak et al., 2020). In the present *in vitro* study, combination of i-PRF with xenogenic BSM significantly affected cell viability and metabolic activity of HOB, however not equally among the different XBSMs. Noteworthy is that material processed at high temperatures (CB + i-PRF) showed two times higher values of cell viability on day 3, when compared to other

i-PRF-XBSM groups. Interestingly, all abovementioned indexes of i-PRF-CB were even higher than those of i-PRF-controls on day 3 and almost the same at most of the later time-points.

Still, it is not fully clear why the sintered XBSM showed better *in vitro* results above all studied materials—either with i-PRF or without. Therefore, further *in vitro* as well as preclinical studies for comparison between different bovine bone substitutes—for example using different amounts of bone substitute as they might have a dose-dependent effective range, using different media or using cells from different donors—are needed.

According to our *in vitro* study, it could be assumed that i-PRF addition to XBSM may have the potential to improve bone regeneration in clinical application. This might be of greatest importance, in particular, in cases with large complex defects or medically compromised patients. Additionally, XBSM sintered in a higher temperature showed an advantage over the XBSM treated in lower temperatures. The knowledge of the materials' advantages leads to a better understanding of the regenerative processes and may improve the industrial production process.

CONCLUSION

XBSM sintered under high temperature showed better HOB viability through the whole period as well metabolic activity on day 7 and 10 when compared to XBSM groups treated at lower temperatures. The same XBSM with addition of i-PRF showed even better HOB viability on day 3 and 7 as well as metabolic activity through the whole period in comparison to other XBSMs combined with i-PRF.

Overall, combination of XBSMs with i-PRF improves HOB viability and metabolic activity (except for one XBSM on day 3), ALP and BMP-2 expression at earlier stages as well as OCN expression at later stages *in vitro*.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

PK, SK, and SB: hypothesis, concept, and methodology. SK, ES, DH, HS, DT, KS, SB, and PK: formal analysis and investigation. SK, BA-N, and PK: writing—original draft preparation. SK, ES, DH, HS, DT, KS, BA-N, SB, and PK: writing—review and editing. PK, KS, and BA-N: supervision. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3 Diskussion (einschließlich Schlussfolgerungen)

In these two studies, a beneficial in vitro effect of i-PRF in combination with an allogenic (ABSM) and a xenogenic bone substitute material (XBSM) on HOB characteristics was evaluated [45, 46]. BSM in combination with i-PRF showed a significantly elevated HOB viability, attachment and proliferation as well as expression of proliferation and differentiation markers when compared to non-i-PRF-BSM. However, XBSM-i-PRF as well as XBSM showed inferiority when compared to the allogenic bone substitute (ABSM) and, to a greater extent, to ABSM-i-PRF in most of the parameters [45]. In conclusion, the obtained data clearly demonstrate the healing-enhancement effect of the PRF when combined with BSM. Another possible approach analyzed by others may represent the use of single growth factors such as VEGF or PDGF to enhance healing [47, 48]. However, due to high costs and strict regulations, the use of single growth factors in addition to BSM is not implemented up-to-date in the clinical pathway. The main prerequisites for ideal BSM are both ease of its handling and application as well as biological characteristics close to autologous bone. Considering that, allograft may be the BSM of choice, due to its unlimited availability, possibility of patient specific customization and its biocompatibility. Generally, ABSM has the potential to induce endochondral bone formation, that is feasible by incorporated BMPs and collagen type I [49-51], although limited in comparison to autologous bone [52]. First, this might be due to storage, processing, and sterilization procedures [53]; second, because of immunologic responses of the recipient [54]. However, on the final stage of incorporation, there are no histologic differences between allogenic and autologous graft [49, 55]. Thus, Soardi et al. have reported allograft to have best outcomes compared to other types of BSM in augmentation of severely atrophied alveolar ridges [56]. Tilaveridis et al. gave evidence for effectiveness of ABSM in sinus augmentation procedures in a retrospective study of critical cases [1]. It was also demonstrated that ABSM is equivalent to autogenous bone in augmentation of single tooth defects [2]. Tong et al. stated an increased effectiveness of demineralized freeze-dried bone in comparison to autologous bone considering implant survival [57]. Besides, Solakoglu et al. gave evidence for a low risk of residual alloimmunization of processed allografts [58].

By comparing different XBSM, the high-sintered group showed beneficial in vitro effects when compared to low-sintered XBSM. Besides, addition of i-PRF to XBSM resulted in a significantly increased biological activity of HOB in most of the cases. The main difference among the four XBSM is the preparation process, namely the temperature [46]. They have a hydroxyapatite phase [59], which causes a good biocompatibility due to similarity with crystalline phase of human bone, high porosity and micro-architecture [60]. Okumura et al. showed evidence that the reason of early osteogenesis on hydroxyapatite lies in the faster initial attachment of HOB [61, 62]. It was also reported that bovine hydroxyapatite materials treated at different

temperatures show significant variation in osteoblastic activity because of changed surface roughness and biological performance (osteoconductivity) [11, 63, 64], which may result in different healing outcomes [11, 13]. In our study, XBSM sintered at high temperatures (cerabone®, CB) showed a significantly increased HOB viability and metabolic activity when compared to other materials processed at lower temperatures. CB is composed of hydroxyapatite with traces of calcium oxide with a porous bone-like morphology [10]. Being sintered at a high temperature (> 1,200° C), it loses all organic compounds. It was reported, that CB presents the highest level of hydrophilicity in comparison to Bio-Oss® (BO) [12]. Besides, in 1,200° C sintered bovine hydroxyapatite, additional traces of NaCaPO4 and CaO were detected, which could result from decomposition of the bone carbonate and could improve HOB reaction [10] as detected in the presented study. Additionally, when considering the carbonate component, the influence of the surface energy of the BSM may also increase initial HOB attachment and proliferation. Thus, the strengthening of the polar components of the dense surface of a bone substitute material may enhance HOB attachment and osteoconduction [65]. The temperature of processing effects the elimination of carbonate content in the bone, which can be only initiated at 400° C and higher [10]. Besides, the high sintering temperature increases crystallinity, subsequently lowers biodegradation rates and increases volume stability [59, 63, 66-68]. On the other hand, it was stated in another study, that high temperature sintering of a XBSM did not affect phase stability, densification behavior, fluid intrusion, and porosity when compared to non-sintered XBSM [69]. In addition, no clinical long-term influence of osseous healing using differently processed bone substitute materials was found. Though, Kapogianni et al. analyzed samples from biopsies 6 months after sinus floor evaluation and after this considerable amount of time in a biological less demanding defect, no differences can be expected [70]. Despite the claim of no organic component, histological analyzes gave evidence for (xenogenic) organic remnants in XBSM treated under lower temperature, which may lead to decreased biocompatibility and osteoconductivity [71]. BO is a carbonated hydroxyapatite, containing water, with porous granulate morphology and nanocristallinity [10]. It is manufactured at a temperature of 300° C, thus, is considered to include residual proteins [14]. In the here presented in vitro investigation, BO showed less distinct results for cell viability and metabolic activity as compared to other XBSMs [46]. These findings are in accordance with other in vitro studies [14, 72]. Sufficient osteogenic cell adhesion a bone substitute material is important for cellular proliferation, differentiation and matrix synthesis. Whereas initial cell attachment is based on unspecific cell-substrate interactions, later cell adhesion displays complex interactions between extracellular ligands and specific cellular receptors with high impact on further intracellular signal transduction [73]. Integrin receptors are transmembrane heterodimers consisting of non-covalently associated α and β sub-units. The sub-units β1 and αv have affinity to extracellular matrix proteins like

fibronectin, collagen, and osteonectin via the RGD tri-peptide sequence [74]. Integrin-mediated outside-in-signaling has been shown to regulate osteogenic cytoskeleton organization and gene expression [75, 76]. Furthermore, during osteoblast / substrate interactions, the expression of these adhesion molecules is modified according to distinct surface characteristics [77].

Recently, it was shown that low speed centrifugation has a direct effect on matrix contents of PRF [39, 78, 79]. Thus, reducing the relative centrifugation force, a new protocol of PRF processing was proposed that can produce a stable (advanced-PRF, A-PRF) or liquid (injectable, i-PRF) membrane [39]. In accordance, the main difference of i-PRF from conventional PRF is the lower speed of preparation and decreased time of centrifugation, that enables to keep some important cellular components in it [24, 39, 40]. Due to these changes in centrifugation time, a significantly increase in leukocyte number in the PRF matrix and an increased expression of growth factors was seen [39, 40, 78, 79]. It was reported that i-PRF shows a significantly higher long-term release of growth factors and a higher fibroblast migration when compared to other platelet concentrates [40]. Considering that, i-PRF was chosen in the present studies to test its synergic effect with different BSM. Although i-PRF has shown success in various clinical implementation [41, 80] there is a lack of reliable data considering preclinical and clinical issues of combination of i-PRF with ABSM and XBSM [81-83]. Previous studies reported that PRF releases autologous growth factors gradually and expresses stronger and more durable effect on proliferation and differentiation of HOB in vitro more than any other platelet concentrates [29, 30]. In the present study, significant differences in between the groups in terms of HOB viability, attachment and proliferation were seen. Here, ABSM-i-PRF showed the most favorable in vitro results in comparison to XBSM-i-PRF, ABSM, and XBSM [45]. In another study addition of i-PRF to high-sintered XBSM resulted in a significantly increased biological activity of HOB in most of the cases [46]. This is in accordance with an animal study by Karayurek et al. that found an increased bone healing in the group with PRF and autografts. Even if the combination of b-tricalcium-phosphate with PRF could not provide superiority in terms of bone regeneration, the immunohistochemical results showed a high expression of proliferation marker such as osteopontin and osteonectin [84]. Some studies investigated the effect of PRF on XBSM [85]. However, there is only low-level evidence of benefits of the combination of PRF with XBSM in terms of graft maturation when transferred in the clinical setting [85-87]. To the best of our knowledge, no other study analyzed the in vitro effect of i-PRF on HOB when combined with ABSM or XBSM. However, the beneficial effect on ABSM was evaluated clinically as Choukron et al. demonstrated a reduction of healing time prior to implant placement after sinus augmentation with freeze-dried ABSM in combination with i-PRF [29, 30]. Addition of PRF to ABSM has shown to enhance bone maturation and to shorten the time span before implant placement [88]. This could be explained by the (minimal)

osteoinductive potential of ABSM which may be further enhanced by platelet concentrates' cellular components and could approximate induction properties of ABSM to autologous bone transplants, providing new approaches in bone regeneration. Interestingly, BMP-2 expression, despite the late HOB passage, showed relatively high expression rate in all groups except for the non-i-PRF groups on day 7 where it failed to show any expression at all [45].

We revealed [46] that ALP, OCN (early as well as late osteogenic differentiation markers), and BMP-2 expression of BO without and with i-PRF was comparably high. In combination with i-PRF, Creos Xenogain® (CX) showed a significantly elevated OCN expression through the whole period in comparison to other groups. However, the results of gene expression markers were inconsistent and it is possible that the inclusion of other gene expression markers such as type I collagen, Runt-related transcription factor 2 (Runx2) and osteopontin would have shown different results. Cell viability/ metabolic activity of CX and MO more or less correlated to each other. MO is produced via low-heat processing of bovine bone, preserving the coarseness of bone with its high porosity. In a recent preclinical in vivo study, MO showed more osteogenic cells as well as more newly formed bone when compared to BO and autogenous bone [89].

In vitro assessment of cell metabolic activity may allow conclusions to be drawn about biocompatibility of biomaterials, and cells that are metabolically active are a precondition for osteoconductivity and osteoinductivity. But it should be clearly understood that in vitro studies still display only a limited part of the general in vivo set-up and there might be a substantial gap between cellular biocompatibility and in vivo models [90, 91]. For example, surface characteristics of hydroxyapatite changes after getting in contact with blood proteins and extracellular matrix components [92]. Thus, monotonous conditions of in vitro studies may distort in vivo results. To the best of our knowledge, there are no in vitro studies on combination of i-PRF with different XBSMs.

In a clinical study, Zhang et al. [86] showed improvement in parameters of bone regeneration when adding PRF to XBSM but there was no statistical significance. In the present in vitro study, combination of i-PRF with XBSM significantly affected cell viability and metabolic activity of HOB, however not equally among the different XBSMs. Noteworthy is that material processed at high temperatures (CB + i-PRF) showed two times higher values of cell viability on day 3, when compared to other i-PRF-XBSM groups. Interestingly, all above mentioned indexes of i-PRF-CB were even higher than those of i-PRF-controls on day 3 and almost the same at most of the later time-points [46]. Still, it is not fully clear why the sintered XBSM showed better in vitro results above all studied materials—either with i-PRF or without. Therefore, further in vitro as well as preclinical studies for comparison between different bovine bone substitutes—for example using different amounts of bone substitute as they might have a dose-dependent effective range, using different media or using cells from different donors—

are needed. According to our in vitro study, it could be assumed that i-PRF addition to XBSM may have the potential to improve bone regeneration in clinical application [46]. This might be of greatest importance, in particular, in cases with large complex defects or medically compromised patients. Additionally, XBSM sintered in a higher temperature showed an advantage over the XBSM treated in lower temperatures. The knowledge of the materials' advantages leads to a better understanding of the regenerative processes and may improve the industrial production process.

The studies suffer from some limitations. First, as described above, these are in vitro pilot studies. Therefore, results should be reevaluated in an animal model and further prospective trials before implementation in the clinical workflow. Second, future studies should also include HOB alone with and without i-PRF to evaluate the effects of the biomaterials and PRF alone. Third, growth factor expression of i-PRF varies intra- and interindividual. Therefore, a cohort study may help to further characterize "optimal" expression levels for future clinical use. Choukroun et al. revealed that the donor-related values of the platelet distribution and the leukocytes number by different protocols of PRF preparation were approximately similar [40]. On the other hand, there is emerging evidence of interindividual differences (age, sex, underlying conditions) concerning mainly the platelet counts of the different donors that seems to correlate with the fibrin network density and released growth factor levels [93]. Here further studies are also needed.

CONCLUSION

I-PRF in combination with ABSM and – to a lesser extent – to XBSM enhances proliferation, cell migration and differentiation of HOB when compared to non-i-PRF-BSM in vitro [45].

XBSM sintered under high temperature showed better HOB viability through the whole period as well metabolic activity on day 7 and 10 when compared to XBSM groups treated at lower temperatures. The same XBSM with addition of i-PRF showed even better HOB viability on day 3 and 7 as well as metabolic activity through the whole period in comparison to other XBSMs combined with i-PRF. Overall, combination of XBSMs with i-PRF improves HOB viability and metabolic activity (except for one XBSM on day 3), ALP and BMP-2 expression at earlier stages as well as OCN expression at later stages in vitro [46]. All above mentioned could ultimately result in a clinical advantage in terms of faster bony healing.

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