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In Vitro Investigation of Jellyfish Collagen as a Tool in Cell Culture and (Bone) Tissue Engineering

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Abstract. *Background/Aim: Jellyfish collagen serves as a competitive alternative to mammalian-sourced collagen in many practical aspects. For instance, jellyfish collagen lacks religious constraints when compared to bovine or porcine sources and promises batch-to-batch consistency. Another advantage is its structural similarity with many mammalian collagen types, providing a biocompatible matrix for different cell types as "collagen type 0". This paper intends to investigate jellyfish collagen (Jellagen®) in two applications. This investigation aims to establish an initial understanding of jellyfish collagen in biotechnology. More specifically, in cell culture and the field of tissue engineering. Materials and Methods: The jellyfish collagen was comparatively tested as a coating material for multi-well plates as one of the most extensively used tools in cell culture and in the form of three-dimensional (3D) scaffolds intended for bone tissue engineering (BTE) applications. Both, the coated well plates and the scaffolds were seeded with fibroblasts and pre-osteoblasts, separately. In vitro cytocompatibility assays in accordance with EN ISO 10993-5/-12 regulations and LIVE-DEAD-stainings were carried out to study the cell viability, cytotoxicity and proliferation of these two cell lines. Results: The results showed that collagen extracted from R. pulmo jellyfish can be an alternative to mammalian-derived collagen. Fibroblasts showed comparable cell viability to the medium control and an increased cell proliferation on the well plates indicating that these coated well plates can be used in cell culture, particularly in biocompatibility studies of biomaterials (as fibroblasts are used in this respective field extensively). The viability of pre-osteoblasts significantly exceeded the medium control in case of the jellyfish 3D scaffolds. Conclusion: These cells exhibited favorable healthy behavior on this marine collagen, suggesting that Jellagen® collagen can be used in studies of (bone) tissue regeneration and especially as scaffolds in BTE. In conclusion, jellyfish collagen provides biocompatibility and adhesive properties for both cell culture and BTE applications.*

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Key Words: Jellyfish collagen, cytocompatibility, biomaterials, well plates, collagen coating, fibroblasts, osteoblasts, marine collagen, tissue engineering scaffolds, bone tissue engineering.

Collagen and its different subtypes makes up to between 25-35% of the protein mass in human beings (1). Thus, collagen provides both structure and strength in the body. Xenogeneic collagen is used as a biomaterial in many medical fields including dentistry, dermatology, traumatology and orthopedics but also as a substrate for cell culture as well as in biomaterial research (2). Most of the collagen used for these applications is derived from bovine or porcine sources (3). Collagen derived from these two sources has been used in research for more than 30 years but has many different disadvantages such as a lack of reproducibility of the extracted collagen due to the inconsistency between the manufacturers and batches (4). This inconsistency is believed to be a result of the complex anatomy of mammals and the inevitable variances in domestication (5, 6). Moreover, there are concerns about the infectivity of the mammalian raw material due to the presence of contaminants such as transmissible spongiform encephalopathies (TSEs), in particular bovine spongiform encephalopathy (BSE) and other slow virus diseases (7-9). These considerations are of particular interest for pharmaceutical and medical applications as well as in research applications.

Jellyfish-derived collagen has recently been discovered as an appropriate collagen alternative (10). Jellyfish-derived collagen is defined as so-called "type 0 collagen" and shows many similarities with mammalian collagen types I, II, III, V und IX (Table I) (2). Furthermore, jellyfish collagen is significantly less expensive and provides an improved carbon footprint, which is

Table I. *Comparison between collagen derived from different sources and their properties (12-15).*

	Collagen type	Anatomy	Immunogenicity	Pathological risks	Religious/individual limitations	Carbon footprint and cost
Bovine	I, II, IV	Complex	High	High	Yes/Possible	High
Porcine	I, III	Complex	Low	High	Yes/Possible	High
Rat	I	Complex	Medium	Low	No/Possible	Low
Jellyfish	0*	Simple	Low	Low	No	Low

*Collagen type 0 is not an identified type of collagen, rather an agreed upon denotation for jellyfish-derived collagen.

of great interest in green biotechnology (Table I) (10). It should be further noted that, to date, 40% of the world's population does not consume any bovine nor porcine products due to religious reasons, or ethical ones at an individual level (11).

Thus, jellyfish collagen – especially collagen extracted from *Rhizostoma pulmo (R. pulmo)* – is still found to be an alternative extracellular matrix protein for a variety of applications in biomedical research (10). For example, *Paradiso et al.* revealed that jellyfish collagen supports ovarian cancer cell line proliferation, confirming its suitability for advanced cell culturing applications (16). Additionally, another recently published manuscript could demonstrate that jellyfish collagen supports the culture of microglia cells in tumor research (17).

Another very important and specific applications of collagen are the *in vitro* analysis of the cyto- or biocompatibility of biomaterials (18). In this context, laboratory multi-well plates are an extensively used tool in research in the fields of biomaterials and tissue engineering (19). Most cultured cells *in vitro* are anchorage-dependent (except for blood and tumoral cells), meaning that for the cell to survive, it must anchor itself to an appropriate extracellular matrix (ECM) or a surface mimicking this ECM (20). Otherwise, growth halts and anoikis, a form of apoptosis, is triggered (21). Well-plates are made of hydrophobic polystyrene that require surface treatment prior to cell culture purposes. Use of natural collagen coatings can provide the missing bioactivity in contrast to other treatment or coating options such as chemical treatments or synthetic coating (*e.g* RGD peptides) (22). Up to date, the natural polymer most often used for well plate coating is collagen and, specifically collagen type I and IV, that is derived from bovine, porcine or rat tail (15). However, jellyfish collagen is of special interest even for *in vitro* research due to its "precursor molecule" characteristics, which should allow the culture of nearly any cell type (23).

In the context of cytocompatibility analysis of biomaterials, the inclusion of collagen as a coating and reference material might be of special interest. Thus, the first aim of the present study was to analyze the suitability of well plates coated with jellyfish collagen extracted from *R. pulmo* (Jellagen®) for standardized testing of the *in vitro* cytocompatibility analyses of biomaterials based on ISO 10993-5/-12 as previously described (24-26).

Another possibility of using jellyfish collagen can be seen in the field of tissue engineering (27). The three main pillars in this field are cells, biological factors and 3D-scaffolds. Biological factors provide a differential pathway to the cells, while scaffolds act as a platform for the cells to attach and grow (27). Choice of cells and growth factors depends on the application. The scaffolds' chemical, biological, structural, and mechanical properties also affect the application. Collagen is one of the most used natural polymers in the field of tissue engineering (28). That is because of its biocompatibility and favorable cell adhesion (3). Thereby, 3D collagen scaffolds should mimic the extracellular matrix by providing a collagen network with a high porosity and high interconnectivity. In this context, it has already been shown that jellyfish-derived collagen scaffolds promote higher cell viabilities (fibroblasts, osteoblasts, epithelial cells and fibrosarcoma cells) compared to mammalian collagen (12, 29). In addition, the present study also aimed to investigate collagen derived from *R. pulmo* as an alternative for mammalian-derived collagen in the field of tissue engineering.

Altogether, both well plates coated with jellyfish collagen and jellyfish collagen-based 3D scaffolds were seeded with L-929 fibroblasts and MC3T3 pre-osteoblasts. Fibroblasts are the most common cells used for primary investigation of biomaterials, and pre-osteoblasts are commonly used for bone tissue studies. *In vitro* assessment was conducted on the extracts of both materials in accordance with the standard protocols in EN ISO 10993-5:2009. This assessment included the measurements of cell viability (XTT assay), cytotoxicity (LDH assay) and cellular proliferation (BrdU assay). Supplemental LIVE-DEAD staining's were performed after coculturing of the well plates and the 3D scaffolds with the L-929 fibroblasts.

Materials and Methods

Jellyfish collagen 3D scaffolds. 1%-EDC cross-linked collagen scaffolds, extracted from the jellyfish species *Rhizostoma pulmo* (*R.*

pulmo), were obtained in sterile 48-well plate (product code: Jellagen® JSM48) from Jellagen® Limited, Cardiff, UK.

Jellyfish collagen-coated well plates. Extracted research grade collagen from the jellyfish species *Rhizotoma pulmo* (*R. pulmo)* was obtained sterile and provided as a pre-coated collagen in 96-wellplates at a 10mg/cm^2 coating concentration (product code: Jellagen®-JCP96W) from Jellagen® Limited, Cardiff, UK.

Cell lines. L9292 fibroblasts and MC3T3 pre-osteoblasts were used for the *in vitro* experiments. The L-929 fibroblasts were purchased from the Leibniz Institute, German Collection of Microorganisms and Cell Cultures GmbH. The MC3T3-E1 cell line was purchased from the American Type Culture Collection.

Extraction procedure of samples. Both the coated well plates and the collagen sponges were incubated with medium for 72 h at 37˚C and 5% CO_2. The extraction of the coated well plates was carried out according to the EN ISO 10993-12:2012. For the negative control, titanium grades 4 and 5 were used on the L-929 cells and titanium grade 4 was used on the MC3T3-E1. For the positive control, RM-A samples were used for both cell lines on both extracts. Blank control with only the corresponding medium was included and subtracted from the photometric values.

Cell seeding. The extracts were seeded with L-929 fibroblasts and MC3T3-E1 pre-osteoblasts, separately. The cell cultures were incubated for 24 h at 37 C and 5% CO_2. For the *in vitro* assays, the extracts were seeded with 1×10^4 cells/100 µl. For the dead-live staining, the substrates were seeded with 2.4×10^5 cells/1 ml.

Effect of extracts on cell viability. XTT (Sodium 3,3'-[1(Phenylamino)Carbonyl]-3,4-Tetrazolium]-3is(4-Methoxy-6-Nitro) Benzene Sulfonic acid Hydrate) assay was executed to measure cell viability of both the L-929 cells and the MC3T3-E1 cells after co-cultivation with the extracts according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). In brief, the electron-coupling reagent was initially combined with the XTT labeling reagent in a ratio of 1:50, Afterwards, 50 µl of this mixture was added to the cells and substrate conversion was quantified after 4 h of incubation under standard cell culture conditions. The measurments included evaluation of the absorbance of 100 µl aliquots in a new 96 well plate by means of a scanning multi-well spectrophotometer (ELISA reader) with filters for 450 and 650 nm (reference wavelengths).

*Effect of extracts on cell proliferatio*n. BrdU (Bromodeoxyuridine/5-Bromo-2'-Deoxyuridine) ELISA assay was executed to measure cell proliferation of both the L-929 cells and the MC3T3-E1 cells after co-cultivation with the extracts according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). In brief, the cells were stained using BrdU for 2 h under cell culture conditions. Afterwards, the cells were fixed at room temperature using FixDenat reagent. Then, the cells were treated with anti-BrdU-peroxidase (POD) antibody for 1 h followed by a washing step (3 times for 5 min). Detection of these immune complexes was possible following a substrate reaction with tetramethyl-benzidine (TMB) for 20 min at room temperature. This step was followed by addition of 25 µl 1 M H_2SO_4 to stop the reaction. Finally, a scanning multi-well spectrophotometer (ELISA reader) with filters for 450 and 690 nm (reference wavelengths) was used for the analysis.

Effect of extracts on cytotoxicity. Lactate dehydrogenase (LDH) assay was executed to measure cell cytotoxicity for both the L-929 cells and the MC3T3-E1 cells co-cultivated with the extracts according to the manufacturer's instructions (BioVision, Milpitas, CA, USA). In brief, 10 µl of the cell supernatants were incubated with 100 µl LDH reactivity solution for 30 min at room temperature and this reaction was stopped using the respective solution. Afterwards, absorbances were analyzed *via* s scanning multi-well spectrophotometer (ELISA reader) with filters for 450 and 650 nm (reference wavelengths).

LIVE-DEAD staining. The specimens and controls were seeded with 2.4×10^5 L-929 fibroblasts in 1 ml medium in each well of 12 well plates (the surface-area/medium ratio was 5.65 cm^2/ml). For the negative control, titanium grades 4 and 5 were used. For the positive control, RM-A samples were used. Assays were carried out after 24 h incubation under cell culture conditions. In order to perform LIVE-DEAD cell staining on the surfaces of the specimens, 60 µl per ml medium propidium iodide (PI) stock solution (50 µg/ml in PBS) and 500 µl per ml medium fresh fluorescein diacetate (FDA) working solution (20 µg/ml in PBS from 5 mg/ml FDA in acetone stock solution) were added to each well. After a brief incubation for 3 minutes at room temperature, specimens were rinsed in prewarmed PBS and were immediately examined with an upright fluorescence microscope (Nikon ECLIPSE Ti-S/L100, Nikon GmbH, Düsseldorf, Germany) equipped with a filter for parallel detection of red and green fluorescence. Photos were taken using a 4×, 10× and 20× objective.

Statistics. The data generated by the different afore-mentioned analysis methods were statistically analyzed by an analysis of variance (ANOVA) combined with a Tukey's multiple comparisons test *via* the GraphPad Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical differences were designated as significant if p-values were less than 0.05 (*$p\leq0.05$), and highly significant if *p*-Values were less than 0.01 (**$p\leq0.01$) or less than 0.001 (***$p\leq0.001$).

Results

Effects of extracts on cell viability. L-929 fibroblasts in the group of the jellyfish collagen-coated well plates exhibited a significantly increased cell viability (**$p<0.01$) compared to the medium control (Figure 1A). Furthermore, in the group of the jellyfish collagen-based 3D scaffolds a highly significant decrease (***$p<0.001$) in cell viability compared to the values in the medium control group was measured (Figure 1A). No significant differences between the viability percentages in both jellyfish collagen groups and the titanium grade samples were measured (Figure 1A). Additionally, the values in all afore-mentioned groups were highly significantly increased (****$p<0.0001$) compared to the positive control group.

In contrast, the cell viability of MC3T3 pre-osteoblasts showed a highly significant increase (****$p<0.0001$) in the

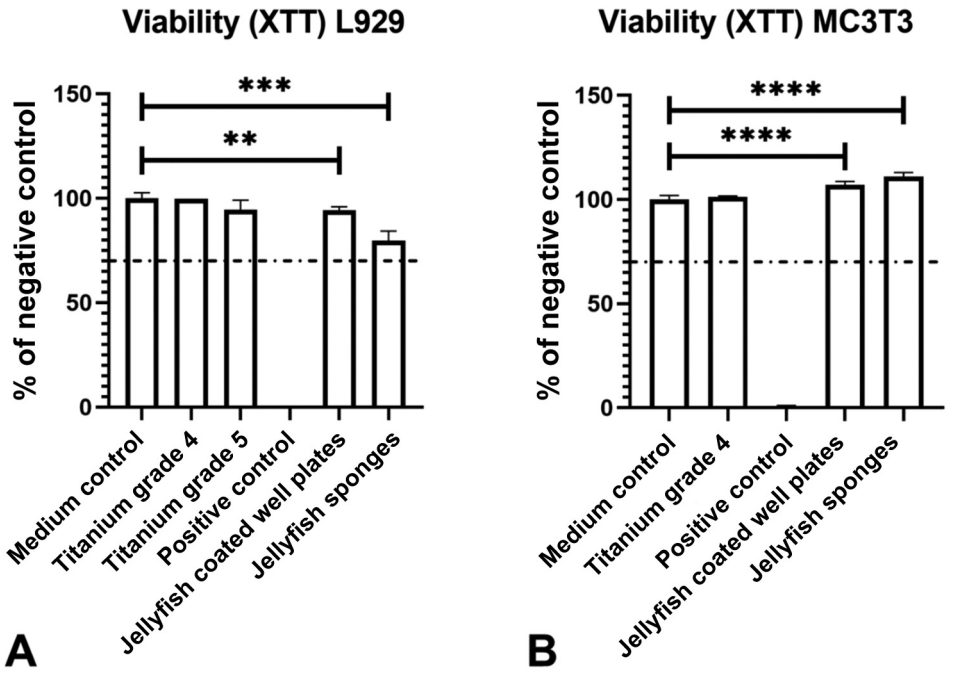

Figure 1. *Photometric levels of XTT (cell viability) assay analyses of (A) L929 fibroblasts and (B) MC3T3 pre-osteoblasts cocultured with extracts from jellyfish collagen-coated well plates and jellyfish collagen-based 3D scaffolds. (**p<0.05, ***p<0.001 and ****p<0.0001).*

group of the jellyfish collagen-coated well plates compared to the medium control values (Figure 1B). Also, in the group of the jellyfish collagen-based 3D scaffolds a highly significantly increased cell viability (****p<0.0001) could be detected (Figure 1B). No significant differences between the viability in both jellyfish collagen groups and the titanium grade 4 sample were measured (Figure 1B). Additionally, the values in all afore-mentioned groups were highly significantly increased (****p<0.0001) compared to the positive control group.

Additionally, a comparison of the viability of both cell lines onto the both jellyfish collagen-based materials showed that the viability of the MC3T3 pre-osteoblasts was highly significantly higher (****p<0.0001) compared to the L929 fibroblasts (Figure 2). In contrast, no significant differences were found in the groups of the medium controls (Figure 2).

Effects of extracts on cytotoxicity. The analysis of the cytotoxicity revealed that the L-929 fibroblasts in the group of the jellyfish collagen-coated well plates exhibited a higher but non-significant LDH increase compared to the values in the medium control (Figure 3A). The values in the group of the jellyfish collagen-based 3D scaffolds were comparable to the values in the medium control group (Figure 3A). Furthermore, no significant differences between the LDH values in both jellyfish collagen groups and the titanium grade samples were

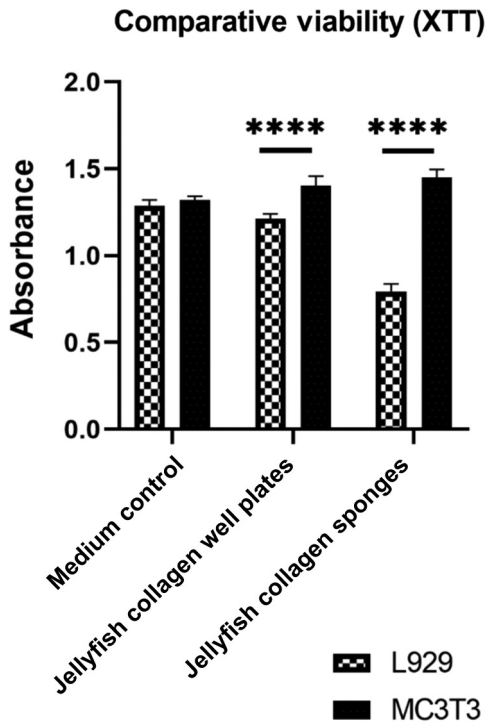

Figure 2. *Comparison of the cell viabilities via XTT assay of L929 fibroblasts and MC3T3 pre-osteoblasts (****p<0.0001).*

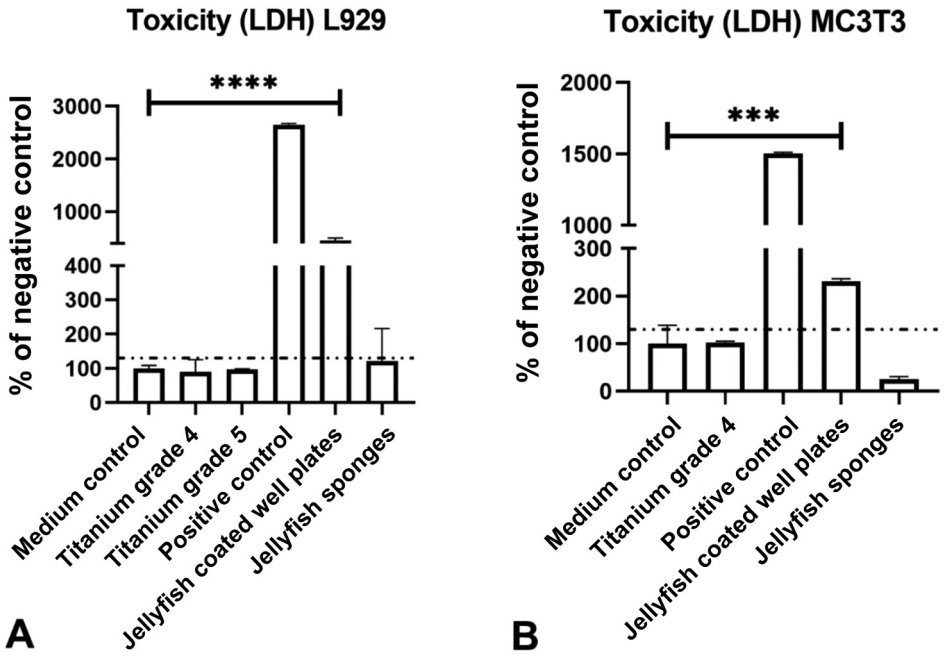

Figure 3. *Photometric levels of LDH (cytotoxicity) assay analyses of (A) L929 fibroblasts and (B) MC3T3 pre-osteoblasts cocultured with extracts from jellyfish collagen-coated well plates and jellyfish collagen-based 3D scaffolds. (***p<0.001 and ****p<0.0001).*

measured (Figure 3A). Additionally, the values in all aforementioned groups were highly significantly decreased (****p<0.0001) compared to the positive control group.

The cytotoxicity analysis also showed that the MC3T3 pre-osteoblasts showed a highly significant LDH increase (***p<0.001) in the group of the jellyfish collagen-coated well plates compared to the medium control values (Figure 3B). Furthermore, the LDH values in the group of the jellyfish collagen-based 3D scaffolds were comparable to that in the medium control group (Figure 3B). Furthermore, no significant differences between the LDH values in both jellyfish collagen groups and the titanium grade sample were detected (Figure 3B). Finally, the values in all aforementioned groups were highly significantly decreased (****p<0.0001) compared to the positive control group (Figure 3B).

Additionally, a comparison of the cytotoxicity of both cell lines onto the both jellyfish collagen-based materials showed that the cytotoxicity of the MC3T3 pre-osteoblasts was lower compared to the L929 fibroblasts but without a statistical significance (Figure 4). No significant differences were found in the groups of the medium controls.

The proliferation analysis of L-929 fibroblasts revealed that a highly significant increase in the proliferation rate (***p<0.001) in the group of the jellyfish collagen-coated well plates was detectable compared to the values in the medium group (Figure 5A). In contrast, a highly significant decrease of proliferation (****p<0.0001) in the group of the jellyfish collagen 3D scaffolds was measured compared to the medium control group. However, no significant differences were found in both jellyfish collagen material groups compared to the titanium reference materials or compared to each other (Figure 5A). Additionally, the values in the positive control group were highly significantly decreased (****p<0.0001) compared to the values in all other study groups (Figure 5A).

The proliferation analysis of MC3T3 pre-osteoblasts showed a highly significant decrease (***p<0.001 and ****p<0.0001) of the proliferation rates in both jellyfish collagen groups compared to the values in the medium control group (Figure 5B). Furthermore, no significant differences between the values in both jellyfish collagen groups and the titanium control group were detected (Figure 5B). Finally, the proliferation values in the positive control group were highly significantly decreased (****p<0.0001) compared to that in all other study groups (Figure 5B).
Additionally, a comparison of the proliferation of both cell lines onto the both jellyfish collagen-based materials showed that the proliferation of the MC3T3 pre-osteoblasts was slightly lower on the collagen-coated well plates (p<0.001) and higher on the collagen-based scaffolds (p<0.0001), compared to the L929 fibroblasts (Figure 6). No significant differences were found in the groups of the medium controls (Figure 6).

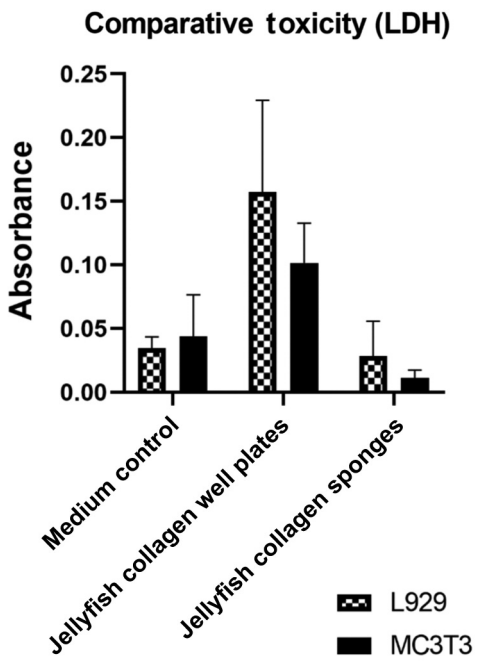

Figure 4. *Comparison of the cytotoxicity via LDH assay of L929 fibroblasts and MC3T3 pre-osteoblasts.*

LIVE-DEAD staining. In the LIVE-DEAD assays, green cells indicate living cells and red cells indicate dead cells. Furthermore, adherent cells appear in a spindle-shaped cell morphology. Both collagen materials exhibited, comparable with the negative controls, green and fluorescent cells (Figure 7). In contrast, only a few vital cells and some red cells could be found in the RM-A group (Figure 7).

Discussion

Collagen has gained enormous importance over recent decades not only as a biomaterial but also for use in biomedical research. In this context, collagen is a necessary coating material used to coat well plates to perform cyto- and biocompatibility studies. It allows a biomimetic growth of nearly all cell types and to obtain valid *in vitro* results that are comparable with the respective *in vivo* microenvironment. Furthermore, collagen is necessary as an extracellular matrix (ECM) component for (bone) tissue engineering studies – especially to obtain the third dimension that is essential for tissue imitation (27). Most of the collagen used for these applications is derived from bovine or porcine sources (3). However, it has been shown that these collagen types have some disadvantages such as a lack of reproducibility (10-12).

Additionally, concerns about the safety of these materials, especially from bovine sources, have recently increased (8, 10). In this context, collagen products have become the focus of several regulatory measurements to reduce the potential risk of transmission of bovine spongiform encephalopathy (BSE) associated with new variant Creutzfeldt-Jakob disease (nvCJD), a fatal neurological disease in humans (8). There are concerns that the purification steps currently used in the process of extracting collagen from animal tissues and bones may not be sufficient to remove the likelihood of infectivity due to the contaminating SE-bearing tissue (*i.e.* brain tissue, *etc*) (8). Raw bovine materials for the extraction of collagen to be incorporated into animal/human food products, pharmaceutical, medical or cosmetic applications are carefully sourced to the U.S. and E.U. (8). The importing of these raw materials halts when the source country is witnessing increased cases of BSE.

Current manufacturing processes of mammalian precursor tissues involve several purification and cleaning steps and may require extensive methods of extraction (10, 30). These tissues are treated in a melting process and the extracted material is subjected to various chemical treatments, including prolonged exposure to strongly acidic or alkaline solutions (30). Numerous purification steps may include washing and filtration and various heat treatments (30). Acid demineralization and liming treatments are used to remove impurities such as non-collagenous proteins and bones must be degreased. Additional washing and filtration steps, ion exchange and other chemical and sterilizing treatments are added to the process with the aim to secure the material. In addition, contamination and impurities may remain after processing, and the resulting collagen product typically needs to be clarified, purified and often further concentrated before usage (31). Altogether, the current extraction methods result in collagen products that are most often a heterogeneous mixture of proteins containing polypeptides with molecular weight distributions of varying ranges (32). It is sometimes necessary to mix different batches of the product to obtain a collagen mixture with physical properties suitable for use in a desired application (33). In addition, there is a need, particularly in the pharmaceutical, cosmetic and (bio-) medical industries, for a collagen source other than those obtained by extraction from mammalian sources.

This gap may be replenished by jellyfish collagen as a cost-effective source of collagen with a lower carbon footprint (15). Jellyfish collagen also fulfills different religious or ethical demands (13, 14). Another important aspect of jellyfish collagen - especially collagen extracted from *Rhizostoma pulmo (R. pulmo)* – is providing similarity with mammalian collagen types I, II, III, V und IX being defined as "type 0 collagen" (2). This means that is of special interest, even for *in vitro* research due to its "precursor molecule" character, which is expected to culture nearly every cell type. Thus, it has already been shown that jellyfish collagen extracted from *R. pulmo* is an alternative extracellular matrix protein substrate for cultivation of a broad variety of cell types like ovarian

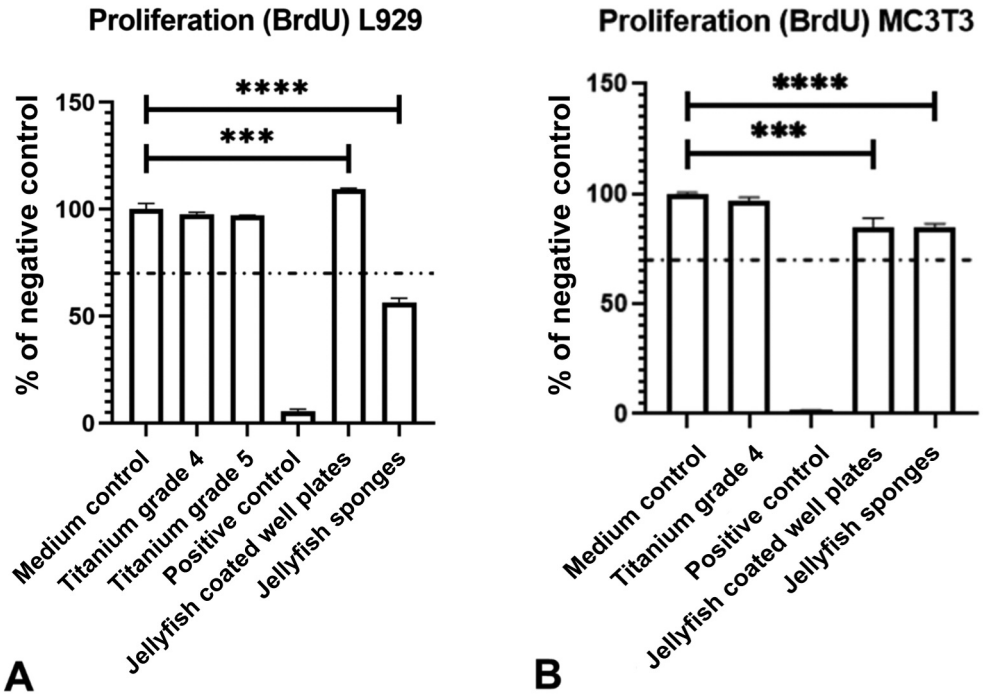

Figure 5. *Photometric levels of BrdU (cell proliferation) of (A) L929 fibroblasts and (B) MC3T3 pre-osteoblasts co-cultured with extracts from jellyfish collagen-coated well plates and jellyfish collagen-based 3D scaffolds (***p<0.001 and ****p<0.0001).*

cancer cells, microglia cells, mesenchymal stem cells (MSCs), fibroblasts, osteoblasts, epithelial cells and fibrosarcoma cells (12, 16, 17, 29, 34).

In the context of the analysis of the cytocompatibility of biomaterials such as bone substitutes, this is also expected to be of special interest for co-culturing materials together with fibroblasts and osteoblasts. Thus, the first aim of the present study was to analyze the suitability of well plates coated with jellyfish collagen extracted from *R. pulmo* for the standardized testing of the *in vitro* cytocompatibility analyses of biomaterials based on ISO 10993-5/-12 (24). In this context, the results of the present study showed that the L-929 fibroblasts that are mainly used for such studies showed a decreased cell viability and an increased cytotoxicity after their cocultivation with extracts from the coated well plates compared to the medium control values. In contrast, it was revealed that the jellyfish collagen coating had a positive influence onto the proliferation behavior of the fibroblasts showing a good cell growth combined with a healthy morphology analyzed *via* LIVE-DEAD-staining. It has to be mentioned that no differences were measured comparing all data in the group of the jellyfish collagen-coated well plates with the control group of the titanium implants, which are known to be particularly biocompatible. Thus, this first partial results show altogether that the well plates coated with jellyfish collagen extracted

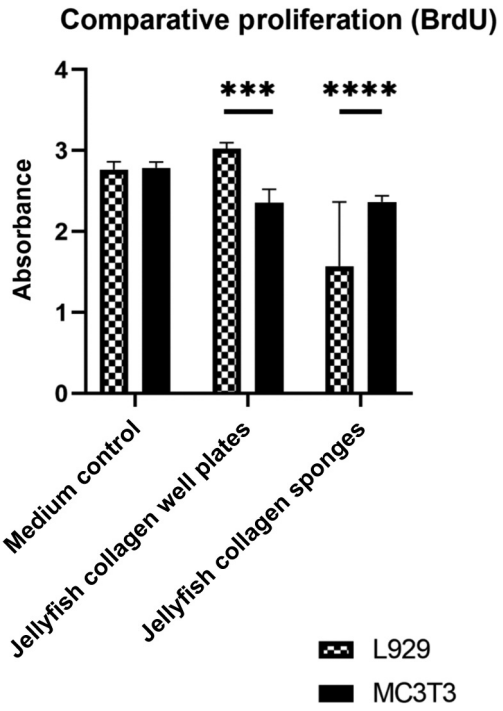

Figure 6. *Comparison of proliferation, via BrdU assay, of L929 fibroblasts and MC3T3 pre-osteoblasts. (***p<0.001, ****p<0.0001).*

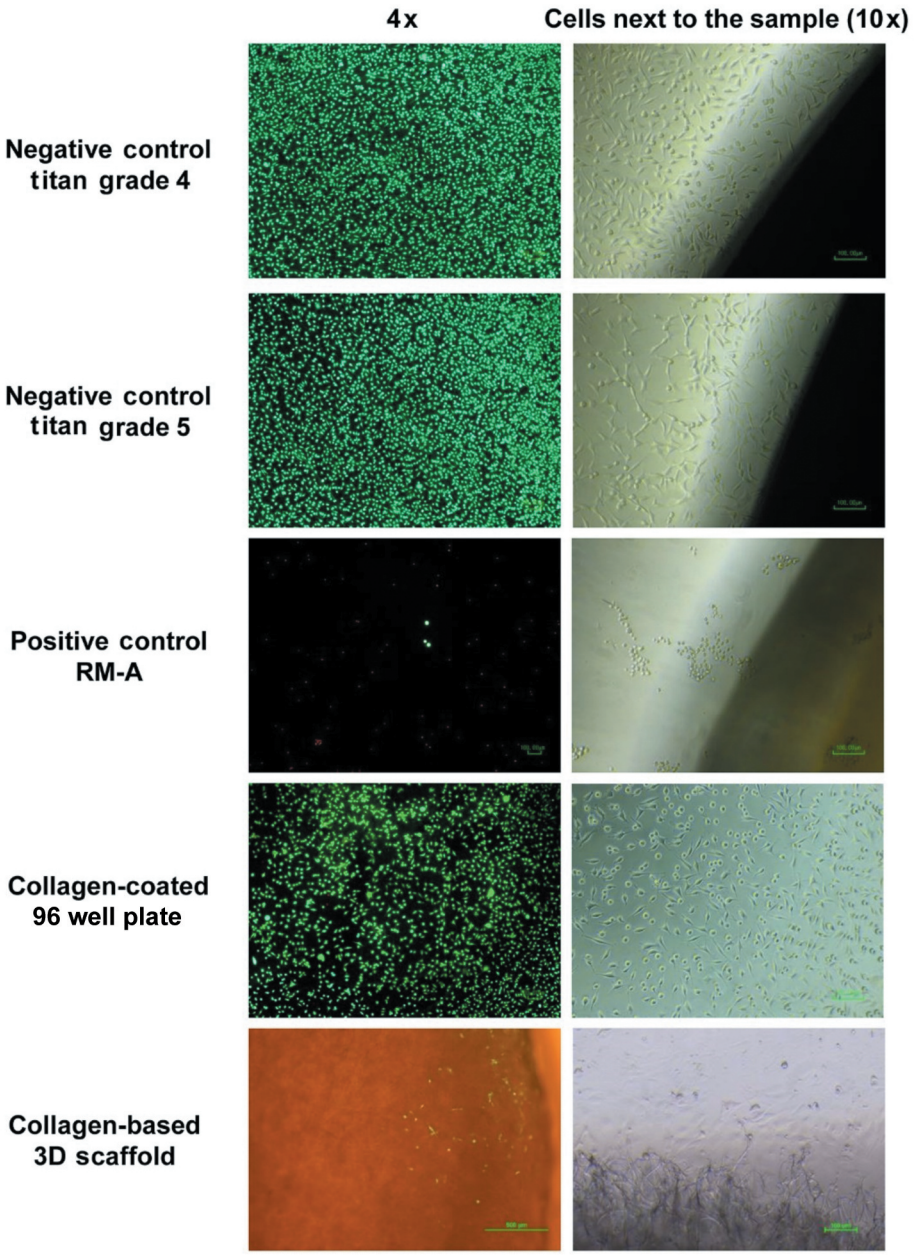

Figure 7. *LIVE-DEAD stainings (left column, 4× magnification) and microscopic images (right column, 10× magnification) of L-929 fibroblasts on the negative controls, titanium Grade 4 and 5 samples, the positive RM-A control, jellyfish collagen coated 96-well plates and jellyfish collagen-based 3D-scaffold after 24 h.*

from *R. pulmo* are fully cytocompatible. It can be concluded that they are fully recommendable as test devices for cytocompatibility analyses of biomaterials.

Interestingly, the results showed that the MC3T3 pre-osteoblasts exhibited an increased cell viability when compared to the viability of the fibroblasts. However, an increased cytotoxicity and a decreased proliferation compared to the medium control values were observed but importantly no differences were measured comparing all data in the group of the jellyfish collagen-coated well plates with the control group of the titanium implants. Taken together, it can also be concluded that the well plates allow for an optimal growth of osteoblasts and are an optimal device for *in vitro* bone tissue studies.

Even though the cytotoxicity levels were slightly increased for both cell lines compared to the controls, it had no significant adversity on the metabolic activities. However, proliferation rate significantly increased with the fibroblasts and significantly decreased with the pre-osteoblasts. This decreased proliferation rate of pre-osteoblasts could be a result of not using an osteogenic differentiation medium (ODM). In this context, it has already been shown by Nishimura *et al*. investigating the effect of ODM on human mesenchymal stem cells (hMSCs) that these cells The hMSCs exhibited significantly higher proliferation rate on ODM compared to regular growth medium (35). The cytotoxicity data are therefore considered to be an artefact and should not be overvalued. In this context, it has to be mentioned that a combination of different assays has been used to analyze the cytocompatibility in this study. This approach has the advantage that the cytocompatibility is determined at different levels and with different reagents. Thus, the results of the present preliminary study suggest that coating multi-well plates with jellyfish-derived collagen can be an alternative tool in cell culture since the viability of the cells is a more important parameter in evaluating the extent of cytocompatibility. Further direct and elongated *in vitro* studies are suggested to investigate the cells morphological behavior when adhering to this coating. Furthermore, there is no study that compares the jellyfish-derived collagen coating to mammalian-derived and synthetic coatings as a tool in cell culture to this day.

Moreover, the analyses revealed that the 3D scaffolds made from jellyfish collagen exhibited dissimilar viability levels on the mouse fibroblasts and pre-osteoblasts. While the L929 viability decreased compared to the medium control, the pre-osteoblast viability increased. Both cell lines exhibited decreased cytotoxicity on the collagen-based scaffolds and when compared to each other, the cell lines had different proliferation rates as well. Furthermore, a significant reduction in proliferation rates for both cell types compared to the medium control were detected. Significant differences were also found regarding cytotoxicity. In this case, no differences were measured when comparing all data in the group of the jellyfish collagen-based 3D scaffolds with the control group of the titanium implants.

Altogether, this data underlines the assumption that these 3D scaffolds could be suitable for application in TE applications and especially for BTE studies, which complies with the *in vivo* results of these scaffolds (2). The decrease in viability of the fibroblasts could also be a result of the chemical cross-linking. These scaffolds are crosslinked with 1% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), which has already shown to adversely impact cell viabilities of different cell types (36, 37). Awang *et al*. studied EDC crosslinking and found the similar slight decrease in viability of fibroblasts (36). Furthermore, the reduced proliferation of the pre-osteoblasts on these scaffolds can also be a consequence of not using ODM, as discussed above.

However, the scaffolds exhibited non-cytotoxicity on both cell lines, confirming that this jellyfish collagen-based scaffold is biocompatible. Further studies on the characteristics (degradation, surface morphology, mechanical properties *etc*.) of these scaffolds should be conducted as the results of this paper suggest that these scaffolds might be complying with the requirements for using scaffolds in BTE. An indication of this assumption is seen in the significant difference of cell viabilities between the two cell lines. Further studies on alkaline phosphate (ALP) levels *in vitro* can verify this assumption. Moreover, direct cell seeding can provide a closer look on the osteopromotive properties and the interconnectivity of these scaffolds. Another interesting topic might be to study the cocultivation of these scaffolds with osteoblasts and endothelial cells. It has already shown *in vivo* that they seem to have an optimal influence on the process of angiogenesis that has been identified as one of the most important molecular processes for tissue regeneration (38).

Collagen makes up 90-95% of the matrix proteins of the human bone tissue, with collagen type I being the most abundant (39). Not only it is responsible for mechanical flexibility and tensile strength in bone, but the collagen molecule also participate in cell-matrix interactions *via* the binding of collagen receptors to cell integrins (1). Hence, collagen as a substrate provides cells (like fibroblasts, pre-osteoblasts and many others) the appropriate micro-environment for adhesion, which is crucial for cellular survival and metabolism as mentioned above. An important advantage of jellyfish collagen is the homology of its collagen type to the most common and abundant collagen types in the human body, one of which is type I. This plays an important factor when considering this marine collagen as an alternative to the current commonly used collagens for cell culture and (bone) tissue engineering. Therefore, adhesion assays on direct cell cultures on different sources of collagen, including jellyfish collagen, can provide further verification and quantification of the promotive capacity of this collagen for cellular adhesion.

As the results above suggest, jellyfish collagen seems to be entering the forefront of xenogeneic collagen. Alongside these results, jellyfish collagen has competing properties that can allow its application in biocompatibility studies to become conventional and extensive. This marine collagen has a lower carbon footprint and costs comparably less, which is intriguing to sustainable and green biotechnology. It also stands as an option for those who follow religious standards that do not allow for the use of bovine or porcine-derived collagen. Altogether, the results of the present study lead to the conclusion that the jellyfish collagen extracted from *R. pulmo* (Jerllagen®) are fully biocompatible and provide optimal properties for both cell culture and TE

applications. It seems that especially the Jellagen®-3D jellyfish collagen scaffolds are suitable tools in studies of bone tissue regeneration and especially as scaffolds in BTE.

Conclusion

This paper investigates the *in vitro* cell behaviors of fibroblasts and pre-osteoblasts on extracts of jellyfish collagen-coated well plates and jellyfish collagen-based 3D scaffolds. The preliminary results suggest that collagen extracted from *R. pulmo* jellyfish (Jellagen®) can be an alternative to mammalian-derived collagen. Fibroblasts showed comparable cell viability to the medium control and seemed to be highly proliferative on the well plates. Suggesting that these coated well plates can be used in cell culture, particularly in biocompatibility studies of biomaterials (as fibroblasts are used in this respective field extensively). The viability of pre-osteoblasts exceeded the medium control by 8%. These cells exhibited favorable healthy behavior on this marine collagen, suggesting that this Jellagen® collagen can be used in studies of bone tissue, specifically as scaffolds in BTE. Studies using ODM with the pre-osteoblasts and measuring the ALP levels can provide further verification of the findings of this paper. Extended periods of direct *in vitro* seeding of both cell lines can be carried out to investigate the morphological behavior of these cells on Jellagen® collagen substrates. Jellagen® seems to provide biocompatible and adhesive properties for cell culture, as well as osteoinductivity and -conductivity needed in BTE.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conceptualization, M.B. and O.J.; methodology, O.J.; software, M.B. and O.J.; validation, M.B., O.J. and S.A.; formal analysis, S.A.; investigation, O.J. and S.A.; resources, M.B. and O.J.; data curation, S.A.; writing – original draft preparation, S.A.; writing – review and editing, S.A. and M.B.; visualization, O.J.; supervision, M.B.; project administration, M.B.; funding acquisition, M.B.

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