A LOW PERCENTAGE OF AUTOLOGOUS SERUM CAN REPLACE BOVINE SERUM TO ENGINEER HUMAN NASAL CARTILAGE

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Abstract

For the generation of cell-based therapeutic products, it would be preferable to avoid the use of animal-derived components. Our study thus aimed at investigating the possibility to replace foetal bovine serum (FBS) with autologous serum (AS) for the engineering of cartilage grafts using expanded human nasal chondrocytes (HNC). HNC isolated from 7 donors were expanded in medium containing 10% FBS or AS at different concentrations (2%, 5% and 10%) and cultured in pellets using serum-free medium or in Hyaff®-11 meshes using medium containing FBS or AS. Tissue forming capacity was assessed histologically (Safranin O), immunohistochemically (type II collagen) and biochemically (glycosaminoglycans – GAG- and DNA). Differences among experimental groups were assessed by Mann Whitney tests. HNC expanded under the different serum conditions proliferated at comparable rates and generated cartilaginous pellets with similar histological appearance and amounts of GAG. Tissues generated by HNC from different donors cultured in Hyaff®-11 had variable quality, but the accumulated GAG amounts were comparable among the different serum conditions. Staining intensity for collagen type II was consistent with GAG deposition. Among the different serum conditions tested, the use of 2% AS resulted in the lowest variability in the GAG contents of generated tissues. In conclusion, a low percentage of AS can replace FBS both during the expansion and differentiation of HNC and reduce the variability in the quality of the resulting engineered cartilage tissues.

Key Words: nasal chondrocytes, chondrogenesis, autologous serum, cartilage tissue engineering

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Introduction

Current clinical practice for the treatment of cartilaginous defects created by trauma, tumor resection, or congenital deformities in the nasal compartment consist on the grafting of autologous cartilage. The main problems associated with such approach are the difficulty to obtain a sufficient amount of implant material and the donor-site morbidity. Tissue engineering offers the possibility of producing autologous cartilage in large quantity, starting from a small tissue biopsy and thus with minimal donor site morbidity. Monolayer expanded human nasal chondrocytes cultured on different scaffolds have been shown to generate grafts with clinically relevant size and with biomechanical and biochemical properties approaching those of native nasal cartilage (Farhadi *et al*., 2006; Rotter *et al*., 2002). Moreover, Rotter *et al*. in their study have not observed age-related difference in the quality of the engineered tissues, thus opening the possibility to apply such technique for a broad patient group.

In the papers mentioned above, foetal bovine serum (FBS) was used for the culture of human nasal chondrocytes. However, the use of animal sera for the generation of grafts for clinical use should be seriously considered since such supplements carry the remote possibility of prion or viral transmission and of immune reaction against animal proteins. Substitution of animal serum with autologous serum would address these issues and bring human cartilage tissue engineering nearer to a safe clinical application.

Several studies reported that human serum promotes the growth of human chondrocytes at similar or superior extent than FBS (Choi *et al*., 1980; Gruber *et al*., 1996; Badrul *et al*., 2004; Chua *et al*., 2004; Tallheden *et al*., 2005; Kamil *et al*., 2007; Chua *et al*., 2007). However such studies focused primarily on the ability of chondrocytes to proliferate in monolayer. Alexander *et al*. were the first to evaluate the effects of human serum on chondrogenesis of tissue-engineered human nasal chondrocytes in 3D culture (Alexander *et al*., 2006). By culturing human nasal chondrocytes in alginate beads, the authors demonstrated that human serum resulted in increased production of cartilaginous extracellular matrix as compared to the FBS control group (Alexander *et al*., 2006). However, for this study a *pool* of human sera was used, therefore the obtained data are not directly applicable to the engineering of *autologous* grafts intended for reimplantation in humans, because of risks of transmission of viral infection.

With the goal of defining clinically-applicable culture conditions for the generation of autologous cell-based chondrogenic grafts for repair of nasal cartilage, we

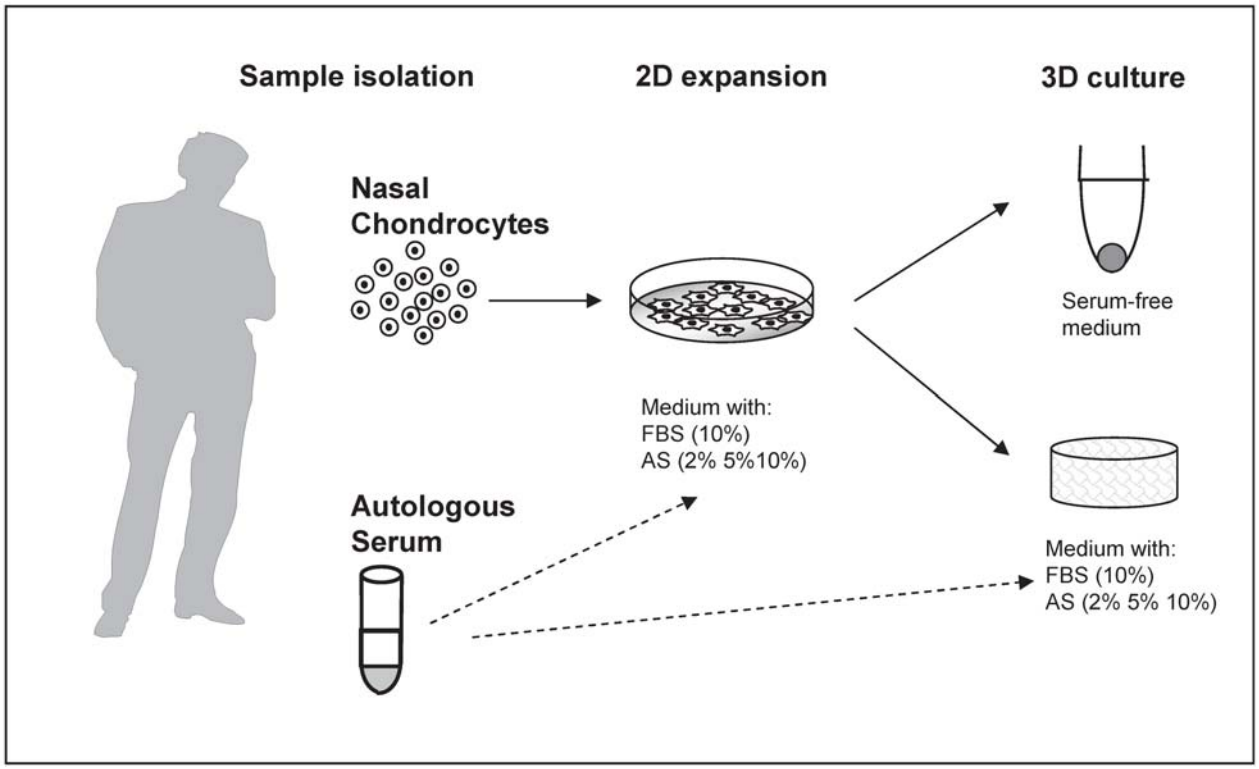

Figure 1. Experimental design. For each donor, nasal chondrocytes were isolated from a cartilage biopsy and autologous serum (AS) separated from blood sample. Chondrocytes were expanded in monolayer in medium containing 10% foetal bovine serum (FBS) or AS at different concentrations (2%, 5% and 10%). Post expanded chondrocytes were then induce to re-differentiate in pellets in serum-free medium or in Hyaff®-11 scaffolds in medium containing the different types and percentages of serum.

determined the effect of autologous serum (AS) on proliferation and post-expansion chondrogenic capacity of human nasal chondrocytes (HNC). Effects of AS at different concentrations (10%, 5% and 2%) were compared to the standard culture condition using 10% FBS.

Materials and Methods

Sample collection

Nasal cartilage biopsies of about 100mg weight were harvested from 7 donors (*donor 1*: 20 years, female; *donor 2*: 21 years, male; *donor 3*: 27 years, female; *donor 4*: 35 years, female; *donor 5*: 41 years, male; *donor 6*: 50 years, female; *donor 7*: 65 years, male) undergoing routine esthetical surgery in accordance with the Local Ethical Committee. Blood (about 50 ml) withdrawn from each patient was collected into serum tubes and incubated at 37°C water bath for one hour to induce its coagulation. Clotted blood was then centrifuged at 3500 rpm for 15 min to separate the serum (about 25 ml).

Chondrocyte isolation and expansion

Cartilage tissues were minced in small pieces and digested with 0.15% type II collagenase (10 mL solution/g tissue) for 22 hours. The isolated chondrocytes were seeded at 10^4 cells/cm^2 with basic medium (Dulbecco's Eagle's Medium containing 4.5 mg/mL D-glucose, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.29 mg/mL L-glutamate) additioned with 10% of foetal bovine serum (FBS) or autologous serum (AS) at different concentrations (2%, 5% and 10%) and further supplemented with 1 ng/mL Transforming Growth Factor β1 (TGFβ1) and 5 ng/mL Fibroblast Growth Factor 2 (both from R&D Systems, 6 Minneapolis, MN). This specific combination of growth factors was previously shown to enhance human nasal chondrocyte proliferation and post-expansion differentiation ability (Tay *et al.*, 2004).

Medium was changed twice a week. When cells were subconfluent (P1), they were detached by sequential treatment with 0.3% type II collagenase, followed by 0.05% trypsin/0.53mM EDTA, replated at $5x10^3$ cells/cm^2 and cultured until they reached one other time the subconfluency (P2). Chondrocytes were subsequently cultivated in pellets or in 3D scaffolds as described below (see also Fig. 1).

Due to the limited amount of human serum obtained, in some cases, it was not possible to culture HNC in pellets or in scaffolds under all the AS conditions.

Pellet culture

To assess the chondrogenic capacity of HNC expanded under the different serum conditions, cells were cultured in pellet using a defined serum-free medium, as previously described (Barbero *et al.*, 2003).

Briefly, HNC were suspended in basic medium supplemented with ITS+1 (Sigma Chemical, St. Louis, MO), 0.1 mM ascorbic acid 2-phosphate, 1.25 mg/ml

human serum albumin, 10^{-7} M dexamethasone and 10 ng/mL TGFβ1. Aliquots of 5 x 10^5 cells/0.5 ml were centrifuged at 1000 rpm for 2 minutes in 1.5 ml polypropylene conical tubes (Sarstedt, Nümbrecht, Germany) to form spherical pellets, which were placed onto a 3D orbital shaker (Bioblock Scientific, Frenkendorf, Switzerland) at 30 rpm. The medium was changed twice per week. Pellets were cultured for 2 weeks and subsequently processed for histological and biochemical analysis as described below.

Culture on porous 3D scaffolds

To investigate how the use of autologous serum at different concentrations could influence the HNC ability to generate 3D cartilaginous tissues using porous scaffolds, post-expanded HNC were induced to re-differentiate in esterified hyaluronic acid non-woven meshes (Hyaff®-11, Fidia Advanced Biopolymers, Abano Terme, Italy), as previously described (Farhadi *et al.*, 2006). Briefly, chondrocytes expanded with 10% FBS or AS at different concentrations (2%, 5% and 10%) were seeded statically on the scaffolds (6 mm diameter, 2 mm thick disks) at a concentration of $4x10^6$ cells/scaffold (corresponding to $7x10^4$ cells/mm^3). Resulting constructs were cultured for 4 weeks in basic medium supplemented with the same serum condition used during expansion and further additioned with 0.1 mM ascorbic acid, 10 mg/ml insulin and 10 ng/ml Transforming Growth Factor-β3. Culture medium was changed twice a week. The resulting cartilaginous tissues were processed for histological and biochemical analyses as described below. At difference from the pellet cultures, serum was added for the culture of HNC in Hyaff®-11, since its presence was preliminarily observed to be required for chondrogenesis in a porous scaffold, probably due to the relatively lower cell density in such environment (data not published).

Analytical methods

Proliferation rate. Proliferation rate was calculated as the ratio of $\log_2(N/N_0)$ to T, where N_0 and N are the numbers of cells respectively at the beginning and at the end of the expansion phase, $\log_2(N/N_0)$ is the number of cell doublings, and T is the time required for the expansion.

Histological and immunohistochemical analysis. Pellets and constructs were fixed in 4% formalin for 24h at 4°C, embedded in paraffin, cross-sectioned (5µm thick) and stained with Safranin O for sulphated glycosaminoglycans (GAG). The "Bern Score" was used to visually score the quality of pellets (Grogan et al., 2006). This assessment takes into consideration (i) the uniformity and intensity of Safranin-O stain, (ii) the distance between cells and amount of matrix produced, and (iii) the cell morphology found within sections. Sections were also processed for immunohistochemistry as previously described (Grogan *et al.*, 2003). Briefly, after pre-treatment with 1 mg/ml chondroitinase, sections were incubated with monoclonal primary antibodies for type II collagen (II-II6B3, Hybridoma Bank, University of Iowa, USA) for 1 h. The slides were then incubated with a biotinylated goat anti-mouse secondary antibody for 35 min followed by StrABC/AP for 45 min (DAKO, Denmark). Bound antibodies were made visible by means of New Fuchsin/Naphthol AS-BI substrate (Sigma Chemical Co., Switzerland).

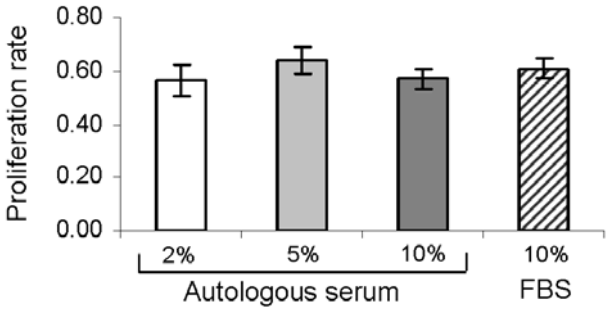

Figure 2. Proliferation rate (number of doublings/day) of HNC expanded in medium containing 10% foetal bovine serum (FBS) or autologous serum (AS) at different concentrations (2%, 5% and 10%). Values are mean ± standard error of the mean (SEM) of measurements obtained from 7 independent experiments. Differences in proliferation rates between AS and FBS were statistically not significant (p values are: 0.31, 0.29 and 0.24 for 2%, 5% and 10% AS).

Biochemical analysis. For determination of GAG and DNA, pellets and constructs were digested with proteinase K (0.5 mL of 1mg/mL protease K in 50mM Tris with 1mM EDTA, 1 mM iodoacetamide and 10 µg/mL pepstatin-A) for 15 hours at 56°C. GAG amounts were measured spectrophotometrically after reaction with dimethyl-methylene blue (Farndale *et al.*, 1986), with chondroitin sulfate as a standard. DNA was measured spectro-fluorometrically using the CyQuant cell proliferation assay Kit (Molecular Probes, Eugene, OR), with calf thymus DNA as a standard (Handley *et al.*, 1995). GAG contents are reported as GAG/DNA.

Statistical analysis. All values are presented as mean ± standard error of measurements from independent experiments with cells from a minimum of 4 different donors. The coefficient of variation (i.e., standard deviation as a percentage of the mean), was used to assess the variability of GAG contents in tissues generated under the different serum conditions. Differences among experimental groups were assessed by Mann Whitney tests, and considered statistically significant with $p<0.05$. Correlation analysis was performed using Pearson's tests.

Results

Proliferation rate of human nasal chondrocytes

HNC expanded in medium containing FBS or AS at the different concentrations tested exhibited a similar elongated fibroblastic morphology (data not shown). HNC proliferated at comparable rates in the different serum conditions (Fig. 2), with no statistically significant differences among groups. HNC cultured in the different

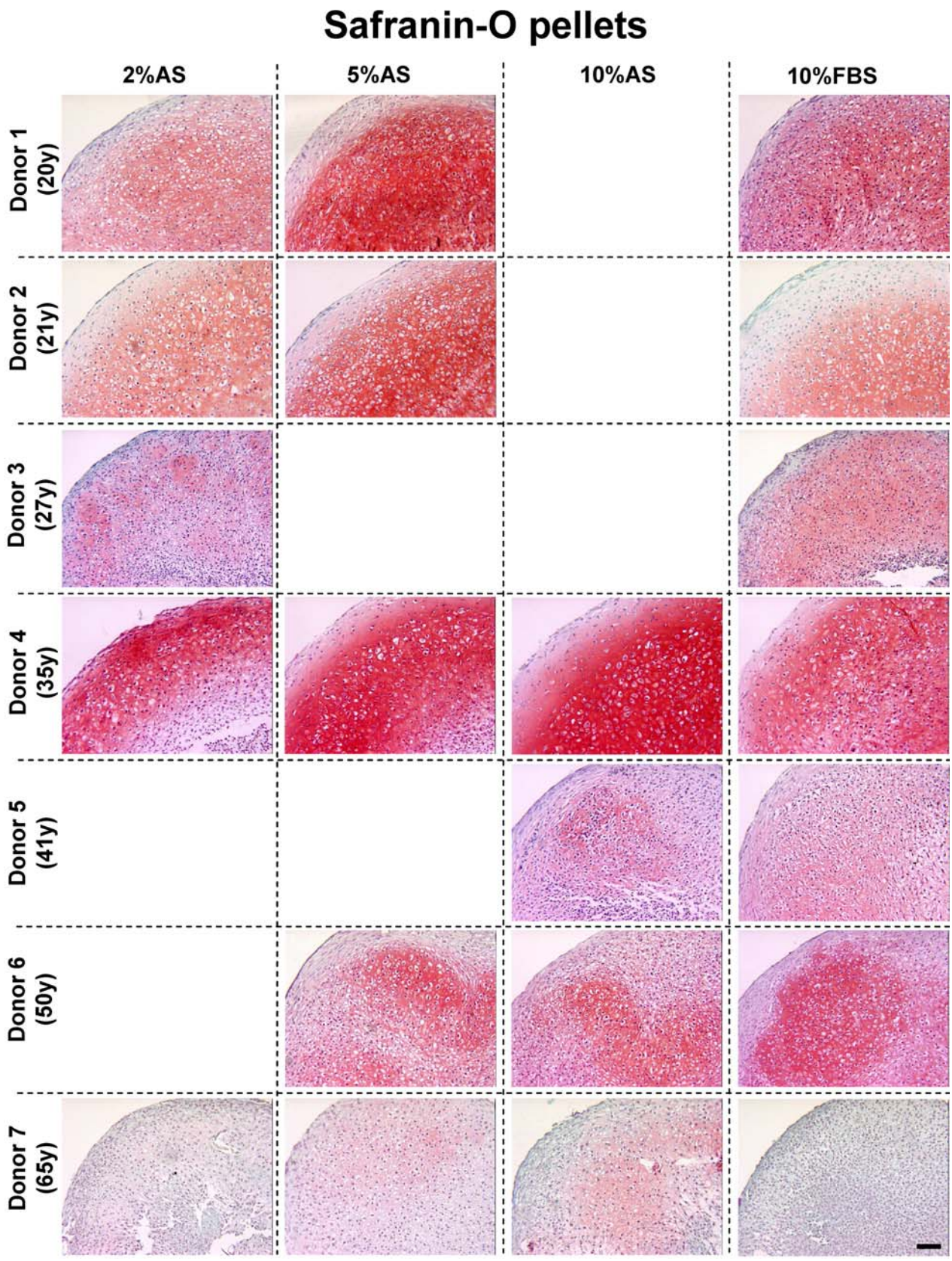

Figure 3. Safranin O staining of representative pellets generated by HNC expanded under the different serum conditions (reported on the top of the panel). During pellet culture, a serum-free medium containing chondrogenic factors was used (see Materials and Methods section). Due to a limited amount of HNC and/or autologous serum, for each donor it was not possible to have the full set of conditions, as evidenced by the empty squares in the panel. Bar = 100 μm.

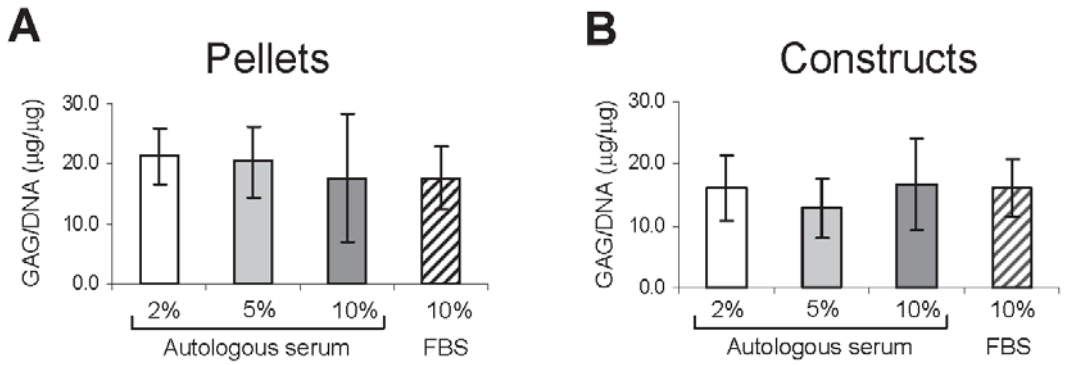

Figure 4. Sulphated glycosaminoglycan (GAG) content of the pellets (**A**) or constructs (**B**), reported as µg GAG/µg DNA. Values are mean ± SEM of measurements obtained from at least 4 independent experiments. Differences in GAG contents between autologous serum (AS) and foetal bovine serum (FBS) were statistically not significant either for HNC cultured in pellets (*p* values: 0.32, 0.37 and 0.50 respectively for 2% AS, 5% AS and 10% AS) and in scaffolds (*p* values: 0.441, 0.31 and 0.40 respectively for 2% AS, 5% AS and 10% AS).

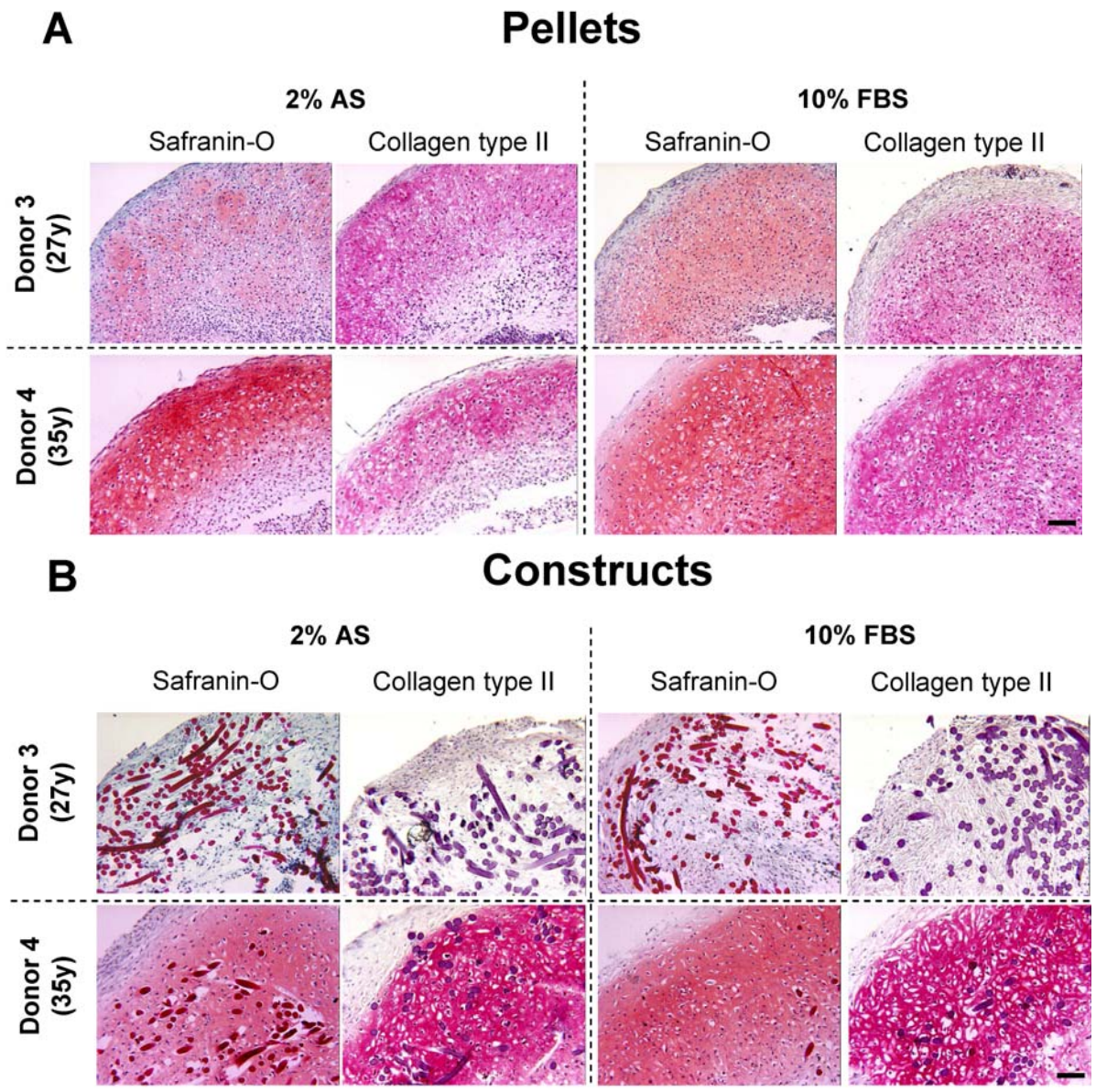

Figure 5. Safranin O and collagen type II stainings of representative pellets (**A**) and construct (**B**) generated by HNC from *donor 3* and *donor 4* cultured with 2% autologous serum (AS) or 10% foetal bovine serum (FBS). Bars = 100 µm

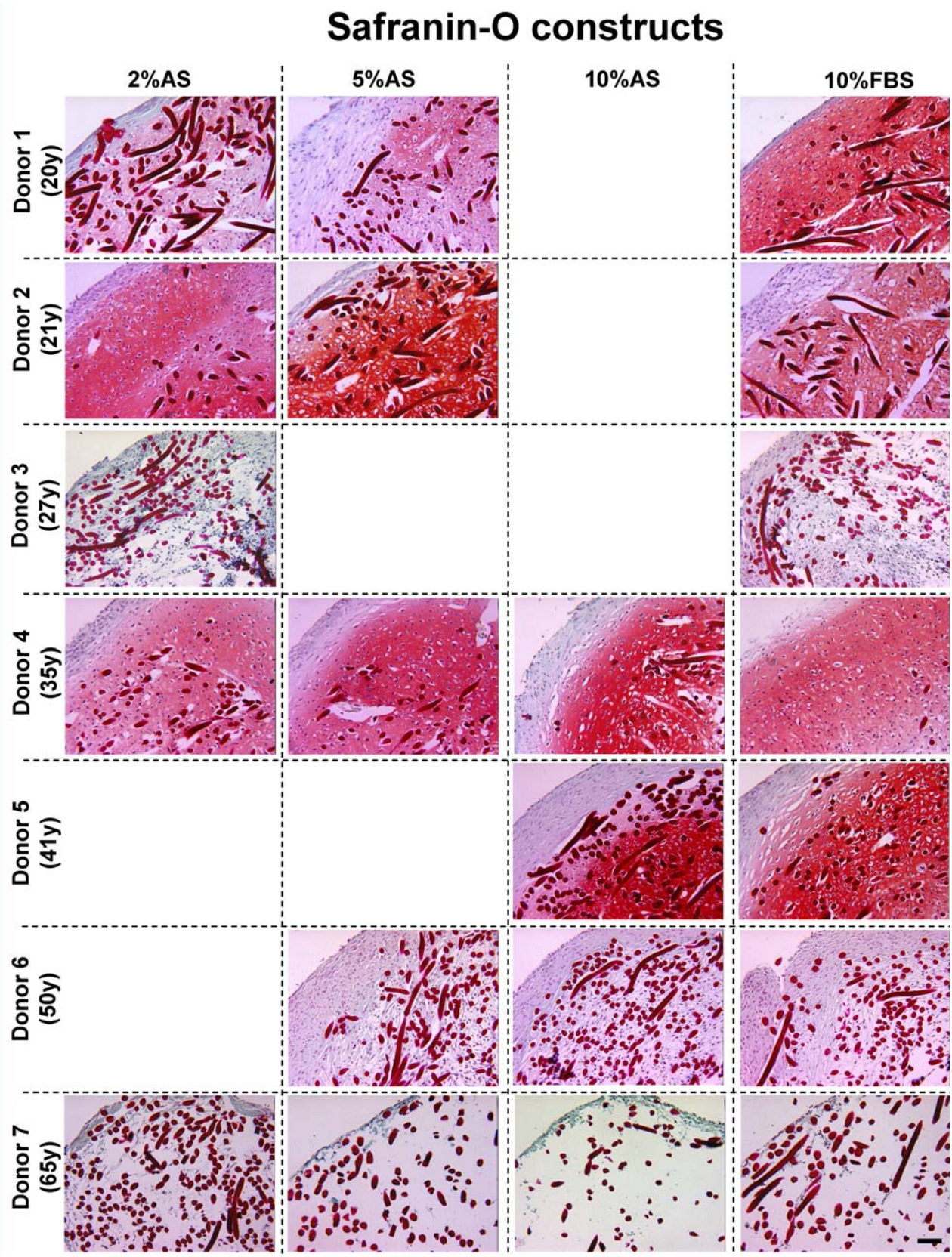

Figure 6. Safranin O staining of representative constructs generated by HNC expanded under the different serum conditions (reported on the top of the panel). During construct culture, the same medium used for cell expansion (i.e., using the corresponding serum condition) was further supplemented with chondrogenic factors (see Materials and Methods section). Due to a limited amount of HNC and/or autologous serum, for each donor it was not possible to have the full set of conditions, as evidenced by the empty squares in the panel. Bar = 100 μm.

conditions underwent a minimum of 8 population doublings in two weeks.

Post-expansion chondrogenic capacity in pellets

After 2 weeks' pellet culture, post-expanded HNC generated cartilaginous tissues whose quality largely differed among the different donors as evidenced by the different intensity of staining for GAG (Fig. 3). However, no systematic differences in tissue quality were observed among pellets generated by HNC expanded with FBS or AS at the different concentrations tested. Visual histological grading of the cartilaginous quality of the pellets according to the "Bern Score" resulted in similar values for pellets generated by HNC expanded with 2% AS, 5% AS, 10% AS or 10% FBS (respectively 6.2±1.2, 6.4±1.0, 5.3±1.3 and 5.6±0.7). Staining appeared to be less intense in pellets generated from the oldest donors as compared to the others, although age-dependent variations in the chondrogenic capacity or in the response to the different serum conditions were beyond the scope of our study.

Biochemical analysis quantitatively confirmed (i) a general large donor-to-donor variability in chondrogenic capacity, evidenced by the large standard error in the GAG content of pellets, and (ii) the similar amount of GAG contents ($p>0.05$) between pellets generated by HNC expanded in the different serum conditions (Fig. 4A). Histological and biochemical findings were consistent, since a statistically significant correlation was found between the GAG/DNA content and the "Bern Score" of pellets ($p<0.001$). Interestingly, a reduced percentage of AS used during the expansion resulted in reduced variability in the GAG contents of pellets, with coefficients of variation (i.e., standard deviation/mean) of 0.49, 0.65, 1.19 and 0.78 respectively for 2% AS, 5% AS, 10% AS and 10% FBS.

Pellets that were more intensely stained for Safranin O were generally also more intensely stained for collagen type II, as assessed by immunohistochemical staining (Fig. 5A) and consistently with a previous study using the same model (Barbero *et al*., 2004).

Tissue-forming capacity in 3D scaffolds

After 4 weeks of culture in scaffolds, the resulting tissues had quality that largely differed between the different donors. Tissues generated by 4 out of 7 donors (*donors 1*, *2*, *4* and *5*) had generally a hyaline-like appearance, with regions intensely stained for GAG. Cells derived from the other donors produced tissues negatively stained for GAG, with fibroblastic cell morphology (*donors 3* and *6*) or large areas of necrosis (*donor 7*). Again, no systematic differences in tissue quality were observed between constructs generated by HNC cultured under the different serum conditions (Fig. 6). In agreement with the histological observations, biochemical analysis demonstrated (i) a generally lower chondrogenesis as compared to pellet cultures, (ii) large donor-to-donor variability in the GAG contents and (iii) similar amounts of GAG contents ($p>0.05$) in constructs generated in the different serum conditions (Fig. 4B). A positive correlation ($p<0.001$) was demonstrated between the GAG/DNA contents of scaffold-based constructs and of pellets generated by cells of the corresponding donors and culture conditions. Similarly to the redifferentiation in pellets, the use of the lowest AS percentage during the culture in scaffold resulted in the lowest variability in the GAG contents of tissues, with coefficients of variations of 0.63, 1.00, 0.89 and 0.77 respectively for 2% AS, 5% AS, 10% AS and 10% FBS.

Constructs that were more intensely stained for Safranin O were generally also more intensely stained for collagen type II, as assessed by immunohistochemical staining (Fig. 5B).

Discussion

In the present study we demonstrated that human nasal chondrocytes (HNC) cultured in medium containing autologous serum (AS), even at low percentage (i.e., 2%), exhibit similar proliferation rates and capacity to re-differentiate and generate cartilaginous tissue than HNC cultured with medium supplemented with 10% foetal bovine serum (FBS).

Previous studies comparing chondrocyte growth with human serum and FBS generally reported superior proliferation rate of chondrocytes in human serum (Choi *et al*., 1980; Gruber *et al*. 1996, Chua *et al*., 2004; Tallheden *et al*., 2005; Chua *et al*., 2007). The discrepancy to our results could be due to a number of different factors, including the origin of the chondrocytes, the presence of additional growth factors in the culture medium and the origin of the serum (autologous or a *pool* of human sera). In any case, considering only 2 passages of cell expansion (i.e., about 8 doublings) and a previously determined yield of about $3.5x10^6$ cells/gram of cartilage tissue (Tay *et al*., 2004), a normal size biopsy (i.e., about 100 mg) would allow (using the conditions of the present study, namely a scaffold thickness of 2 mm and a cell density of $7x10^4$ cells/mm^3) to engineer a graft of about 6 cm^2 size.

In order to assess whether the chondrogenic capacity of HNC could have been influenced by the different serum conditions used during the expansion phase, HNC were then cultured in macromass pellet cultures. Our results indicated that chondrocytes expanded with FBS and AS (even when used at the lowest concentration) exhibited comparable chondrogenic capacity. Similarly, human articular chondrocytes expanded with different concentration of a *pool* of human sera (e.g.: 1%, 5% and 10%) have been shown to generate pellets of comparable quality (Francioli *et al*., 2007).

The influence of autologous serum in modulating the capacity of HNC to generate cartilaginous tissues was also assessed for the culture in 3D porous scaffolds. HNC cultured in Hyaff®-11 meshes with FBS or with the different AS concentration produced tissues with comparable quality and GAG contents. In a previous study, Alexander *et al.* (Alexander *et al.*, 2006) used different concentrations of a *pool* of human sera for the expansion and the 3D differentiation of nasal chondrocytes in alginate

beads. They reported that chondrogenesis of HNC was higher with 10% AS as compared to 10% FBS and 2% AS. The difference between these results and our finding may be related to the different type of human serum used (*autologous vs. pooled* human serum), different 3D culture conditions (scaffold *vs.* gel) and/or duration of the culture (2 *vs.* 4 weeks).

The use of AS at low percentages would be favourable not only to reduce the volume of blood from the patient but also to reduce the dependence of the culture to a non-standardized factor. Indeed, we have demonstrated that a reduced concentration of AS in the culture medium supported a reduced variability in the accumulation of GAG. Despite the improved reproducibility of the result, it should be pointed out that still great differences in the quality of engineered tissue were found even when 2% of AS was used, potentially effecting the outcome of cartilage repair approaches based on grafting of engineered tissues. Thus, our findings point out the importance of the identification of means of predicting and possibly overcoming biological variations in different chondrocyte preparations (Dell'Accio *et al*., 2001; Grogan *et al*., 2007).

In our study, specific growth factors in addition to AS or FBS have been used during the phase of monolayer expansion to enhance the extent of cell proliferation of HNC and during the phase of 3D culture to induce cells to re-differentiate and produce cartilage specific matrix. Therefore, this work could be extended by testing autologous platelet-rich plasma, previously used to stimulate the proliferation and differentiation of chondrocytes (Gaissmaier *et al*., 2005; Akeda *et al*., 2006), to replace both serum and growth factors.

In conclusion, a low percentage of AS can be used for both the expansion and differentiation of HNC, supporting the formation of cartilaginous tissues in the absence of FBS and thus possibly facilitating clinical translation of engineered cartilage constructs for nasal reconstruction. Moreover, considering the recent reports on the responsiveness of HNC to joint-specific regimes of physical loading (Candrian *et al*., 2008), the relevance of the present study could be extended toward the safe culture of HNC for the treatment of articular cartilage defects.

Acknowledgements

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Discussion with Reviewers

S. Grad: The chondrogenic capacity of cells from different donors is known to vary substantially. However, although the outcome of the pellet and scaffold cultures cannot be directly compared due to different culture conditions and times, it may be expected that identical cell populations would behave similarly in pellet and scaffold culture. This does not seem to be the case for all donors. There are even cell populations that stain more intensely in scaffolds than in pellet culture, although generally there is a trend for more matrix accumulation in the pellets. Thus the chondrogenic capacity appears not only to depend on the cell population but also on the culture conditions, which further complicates the identification of markers of *in vitro* chondrogenic capacity. What might be the reason(s) for this different behaviour in different culture, and what are the consequences from the authors' point of view?

Authors: The presented results as well as other unpublished results obtained by our group indicate that chondrogenesis in pellet culture is generally superior to that in scaffold culture, probably due to a higher cell density, which is known to play a critical role in early stages of cartilage formation. With the exception of one donor (*donor 5*), where cells cultured in scaffold accumulated more GAG as compared to those cultured in pellets, our results overall reflect the trend outlined above. As a quantitative confirmation of the qualitative observations, we have now demonstrated a statistically significant correlation (Pearson's test, $p<0.001$) between the GAG/DNA of scaffold-based constructs and of pellets generated by cells of the corresponding donors and culture conditions. Due to the relevance of the raised issue, the result has now been introduced in the text (*penultimate paragraph of Results section*). Based on our findings, we ultimately do feel that it is possible to identify markers of chondrogenic capacity which are valid across different *in vitro* model systems.

S. Grad: It is surprising that there is no difference in cell proliferation among the different serum groups. This may lead to the conclusion that the growth factor supplements added mainly account for the cell growth and that even less serum or, alternatively, serum-free conditions would result in similar proliferation rates. In addition, the finding that a lower percentage of serum, i.e. of undefined components, results in less variation in the outcome suggests that autologous serum may affect the cells either positively or negatively during expansion. Several studies have shown the feasibility of serum-free conditions for culture and expansion of human chondrocytes. Were serum-free media also taken into consideration?

Authors: The Reviewer is absolutely correct in highlighting that in our system the presence of growth factors plays a critical role, likely larger than the concentration of serum. This is also in line with a recent publication from our group, specifically addressing the raised issue (Francioli *et al*., 2007; text reference).

Regarding the use of serum free medium for the culture of chondrocytes, it is true that several studies have proved the feasibility of the concept, but some aspects are not yet fully sorted out. In particular, the initial phase of chondrocyte adhesion critically requires serum or serum components, and the process of cell differentiation in 3D scaffolds – likely because of the limited initial cell density – hardly proceeds in the absence of serum. In our direct experience, the development of a completely serum free system for the engineering of cartilage tissues using human chondrocytes remains a challenge.

M. Stoddart: why did the authors use TGFβ1 in pellets but TGFβ3 in scaffolds?

Authors: For the differentiation in pellets and in scaffolds we decided to use culture media established in previous studies, respectively Tay *et al*. (2004; text reference) and Farhadi *et al*. (2006; text reference). As a matter of fact, the media formulations differ not only in the TGFβ isoforms, but also in many other components (e.g., absence or presence of serum). This was done on purpose, in order to confirm the result using two diverse model systems, and thus demonstrating that the outcome is not strictly dependent on a specific medium composition.

M. Stoddart: Normally an increase in serum concentration is associated with an increase in final GAG content, whereas in this work that does not seem to be the case. Can you offer any suggestions as to why 2% autologous serum results in the same GAG/DNA as 10% autologous serum?

Authors: The literature in this field is quite controversial; therefore it is not really obvious whether a higher percentage of serum during the 3D culture of chondrocytes is associated with an enhanced extent of chondrogenesis. Indeed, some groups claim that absence of serum in high density cultures supports more efficient chondrocyte redifferentiation. Moreover, it must be highlighted that in our experiments the effect of using different percentages of serum, at least during the expansion phase, is masked by the use of growth factors in the medium, which – as mentioned in the discussion with S. Grad – apparently reduce chondrocyte dependence from serum.