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Endotoxins affect bioactivity of chitosan derivatives in cultures of bone marrow-derived human mesenchymal stem cells

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ABSTRACT

Biomaterials research has been expanding over the last decade, in part to provide improved medical devices for the treatment of orthopedic tissue injuries. In the quest to provide the best performance combined with low cost for medical implants, an increasing number of non-chemists have entered the field of biomaterials research without the profound knowledge of chemistry needed to understand the complex interaction mechanisms and characteristics of natural substances. Likewise, non-biologists often lack understanding when it comes to the presence of the contaminating biota frequently found in natural substances. This lack of knowledge by researchers in the field, combined with sensitive in vitro cell-based assays, can lead to inaccurate evaluation of biomaterials. Hence, there should be both an active effort to assemble multi-disciplinary teams and a genuine concern for the possible effects of contamination on in vitro assays. Here, we show that the presence of bacterial endotoxins in chitosan derivatives can result in false-positive results, profoundly altering product performance in in vitro assays. False-positive results through uncritical use of natural substances in vitro can be avoided by proper endotoxin testing and careful evaluation of cytokine secretion patterns.

1. Introduction

Clinical treatment of orthopedic tissue injuries often involves the use of metal implants coated with bioactive materials to improve osseointegration and performance at the bone–biomaterial interface [1]. One of the materials used for this purpose is chitosan, the partly deacetylated form of chitin [2]. Flexible molding abilities, fungistatic and bacteriostatic properties, and recent reports on the positive influence of chitosan on osteogenesis in vitro and in vivo make this polymer a promising candidate for use in regenerative medicine [3–5]. The cationic charge of the polymer, introduced by the removal of the N-acetyl group in the deacetylation process, accounts for its interaction with negatively charged cytokines and growth factors [4]. However, the positive charge also predisposes chitosan to interaction with harmful endotoxins derived from Gram-negative bacteria, based on the formation of hydrogen bonds and electrostatic interactions [6,7]. In fact, chitosan so strongly binds to endotoxins that it was used for the removal of endotoxins from medical preparations before its introduction into biomaterials research and use in clinical applications [8].

Endotoxins are located in the outer cell membrane of Gram-negative bacteria and are released upon cell death, growth and division. Endotoxins and lipopolysaccharide (LPS) are often used as synonyms, even though LPS is only the toxicity-inducing component in endotoxins [9]; here, they are used interchangeably. Endotoxins signal the presence of bacteria to eukaryotic cells. The building blocks of endotoxins include a core oligosaccharide, an O-antigen and a lipid A component, which is the main factor contributing to the induction of immune responses in the host [10]. One of the first events in sensing Gram-negative pathogens and endotoxins is based on the activation of Toll-like receptor 4 (TLR 4) and its interaction with co-modulators, such as MD2 and CD14 [11]. The TLR group consists of receptors that play an important role in innate immunity, and binding of their ligands activates signaling cascades that lead to the secretion of various cytokines and chemokines [11].

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Chitinase-like proteins, e.g. YKL-40 and YKL-39, have putative roles in defense mechanisms against chitin-containing particles, indicating the presence of invading microorganisms, and participate in tissue remodeling and inflammation [12]. Whether activation of immune responses and tissue remodeling are affected by the proteins themselves or by secondary induction of cytokines and growth factors remains unknown [13]. Up-regulation of *YKL-40* in inflammatory and degenerative diseases, such as rheumatoid arthritis, osteoarthritis and certain cancers, suggests that the YKL-40 protein acts in the prevention of damage to the extracellular matrix by reducing the effect of proinflammatory cytokines [14]. Not only is the induction of *YKL-40* dependent on the transcription factor nuclear factor kappa Beta (NF-κB), the main transcriptional pathway activated by the Toll-like receptors, but crystallography studies have also revealed that the enzyme binds chitin and chitooligosaccharides (ChOS) with high affinity [15,16].

Endotoxins are known for their effect on cellular functions, including the stimulation of cytokine secretion, activation of monocytes and macrophages, and an increase in bone turnover in vivo [17,18]. In high concentrations, LPS can cause septic shock and acute renal failure in humans [17]. Therefore, in clinical practice strict regulations are in place to decrease the risk of endotoxin contamination in medical preparations. However, during the in vitro evaluation of biomaterials for clinical applications, endotoxin testing is often neglected. This negligence may ultimately result in erroneous interpretation of bioactivity, posing the risk for undesired health complications during subsequent clinical trials.

Here, we show that the presence of bacterial endotoxins ($10\ ng\ ml^{-1}$) in chitosan derivatives can result in false-positive results, profoundly altering bioactivity in in vitro assays. False-positive results through uncritical use of natural substances in vitro can be avoided by proper endotoxin testing and careful evaluation of cytokine secretion patterns. We intend to raise awareness in the field of biomaterials research to the threat of endotoxin contamination in natural substances, and the resulting discrepancies between in vitro and in vivo studies.

2. Materials and methods

2.1. Production of ChOS

Chitin Flakes (Primex, Iceland) were deacetylated in 50 wt.% aqueous NaOH at 60 °C for 40 min at 25 rpm. The suspension was washed in flowing cold water at 6 °C for 10–12 min in double bag cheesecloth. The resulting chitosan was mixed with water and the pH adjusted to 3.8 with 30% HCl. Degradation of chitosan was performed using chitinase from *Penicillium* species (750 chitinolytic units per g) for 22 h at 25 °C at 50 rpm. ChOS were then separated by ultrafiltration with a Helicon SS50 spiral-wound ultrafiltration membrane (PTGC, 10 kDa cut-off, Millipore, USA) using tangential flow in a Millipore PUF-200-FG pilot module. The filtrate was desalted and subjected to a 1 kDa cut-off Helicon SS50 membrane using the same module. The volume of filtrate was kept constant by the addition of deionized water until oligosaccharides with a degree of polymerization (DP) of 1–3 comprised less than 10% of the total composition of ChOS, as determined by high-performance liquid chromatography (HPLC; HP-SEC with a TSK-oligo column, TosoHaas, Japan).

Before use in cell culture systems, all ChOS were subjected to endotoxin cleaning using Detoxi-Gel Endotoxin Removing Gel (Thermo Scientific, USA). Successful removal of endotoxins was assessed with the PyroGene Recombinant Factor C Endotoxin Detection System (Lonza, USA). For comparison, sample batches were also analyzed by Lonza (Verviers, Belgium) using the same protocol.

2.2. Characterization of ChOS

HP-SEC in a Beckman Gold System with a TSK-oligo column and 5 mM ammonium hydroxide (pH 10.0) as eluent at a flow rate of $0.5\ ml\ min^{-1}$ was used to determine the distribution and quantity of different degree of polymerization oligomers. Twenty microliters of $10\ mg\ ml^{-1}$ ChOS solution was injected and analyzed with an ultraviolet detector at 205 nm. Ethanol served as an internal reference for the inclusion volume and Beckman Gold analysis software was used for peak analysis.

The degree of deacetylation was analyzed using ^{1}H-nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) analysis. ^{1}H-NMR samples were measured in a Bruker AVANCE 400 spectrometer (Bruker Biospin GmbH, Germany) at 400.14 MHz at 298°K. Measurements were performed without water suppression in either D_2O or D_2O/DCl (deuterium chloride) as solvent. The sample concentration was $20–25\ mg\ ml^{-1}$ and the N-acetyl peak was used as an internal reference. The degree of acetylation was calculated using the combined integrals of the proton peaks. IR measurements were performed in an AVTAR 370 FTIR instrument (Thermo Nicolet Corporation, USA). For this, 2–5 mg of samples was thoroughly mixed with KBr and then pressed into pellets with a Specac compressor (Specac Inc. USA).

2.3. Cell culture

Cell culture experiments were carried out with human bone marrow-derived mesenchymal stem cells (Lonza, Switzerland) in DMEM/F12 medium (Gibco, USA) supplemented with penicillin/streptomycin (Invitrogen, USA) and 10% MSC-approved fetal calf serum (Stem Cell, Canada) at 37 °C, 5% CO_2 and 95% humidity. To induce osteogenic differentiation, the basal expansion medium was switched to Differentiation Basal Medium Osteogenic (Lonza) supplemented with dexamethasone, ascorbate, L-glutamine, streptomycin/penicillin, β-glycerophosphate and MSC growth supplement, and 4000 cells cm^{-2} were seeded on vacuum gas plasma-treated tissue culture plastic. Three independent donors were used to determine the effect of $10\ ng\ ml^{-1}$ LPS (Sigma Aldrich Inc., USA), $400\ \mu g\ ml^{-1}$ ChOS (Genis ehf, Iceland) and $10\ ng\ ml^{-1}$ LPS in combination with $400\ \mu g\ ml^{-1}$ ChOS. Proliferation and viability was assessed using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay (ATCC Bioproducts, Sweden) following the manufacturer's instructions.

2.4. Analysis of gene expression

RNA isolation was performed using a Quiagen BioRobot workstation (Quiagen, Germany) and the EZ-1 RNA Cell Mini Kit (Quiagen). Samples from osteogenic differentiation experiments were homogenized in a FastPrep 24-instrument (MP Biomedicals, USA) using Lysing Matrix D tubes (MP Biomedicals) containing 1.4 mm ceramic spheres before RNA isolation. RNA was transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and gene expression of selected genes was quantified in a 7500 Real Time PCR System (Applied Biosystems) and analyzed using GenEx 5.3.2.13 software (MultiD, Sweden). Samples were analyzed at least in duplicate for each of the three donors. *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) was used as the internal reference gene. Taqman assays used during this study included: *YKL-40* (Hs00609691_m1), *TLR2* (Hs00152932_m1), *TLR3* (Hs00152933_m1), *TLR4* (Hs00152939_m1), *COL1A2* (collagen type I; Hs01028970_m1), *ALP* (Alkaline phosphatase; Hs01029141_g1), *OPN* (osteopontin; Hs00167093_m1) and *RUNX-2* (runt-related transcription factor 2; Hs00231692_m1).

2.5. Validation of osteogenic differentiation

Osteogenic differentiation was evaluated by Alizarin Red staining and subsequent quantitation using 10% cetylpyridinium chloride (Sigma Aldrich Inc.), and validated by von Kossa staining, following standard protocols [19]. Alkaline phosphatase activity was determined in cell lysates by adding p-nitrophenyl phosphate solution (Sigma Aldrich Inc.) and measuring the optical density at 405 nm in a MultiSkan spectrometer. Alkaline phosphatase activity was calculated as nmol of p-nitrophenol per min following the general Beer–Lambert law.

2.6. Polystyrene beads cytokine assay

Secretion of eight cytokines into cell culture medium supernatants was determined using a custom-designed Luminex 8Plex Human Cytokine assay (Panomics, USA). The cytokines determined were IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p(40), RANTES and TNF-α with a limit of detection of 1 pg ml^{-1} in medium supernatant.

2.7. YKL-40 enzyme-linked immunosorbent assay

YKL-40 protein concentration in medium supernatants was analyzed with MicroVue YKL-40 enzyme immunoassay (Quidel, USA). The limit of detection was 5.4 ng ml^{-1} and the assay was performed according to the manufacturer's instructions.

2.8. Statistical analysis

Data are presented as mean ± standard error. Statistical analysis was performed using Prism 5.01 software (GraphPad Software Inc., USA) and for quantitative real-time polymerase chain reaction (qPCR) analysis using GenEx 5.3.2.13 software. One-, two- and three-way analyses of variance (ANOVAs) were used to evaluate the effect of endotoxin contamination in ChOS biomaterials. For experiments other than qPCR, one-way ANOVA was used to evaluate the effect of endotoxin contamination. Student's *t*-test was used for direct comparison between two groups; $p < 0.05$ was considered statistically significant. Three independent donors were used in all experiments.

3. Results

3.1. Characterization of ChOS

ChOS preparations were analyzed for degree of acetylation, ash content, solubility and appearance, and are summarized in Table 1. The degree of acetylation was calculated using the combined integrals of the proton peaks (Supplementary Fig. 1). The integral of the protons H-2 (GluNAc), H-3, H-4, H-5, H-6 and H-6′, belonging to the sugar backbone of ChOS, were found to be at δ 3.3–3.9 ppm and H-2 (GluN) was found at δ 2.7 ppm (for D_2O/DCl as solvent at δ 3.2 ppm). The integrals of these protons were compared to the integral for the N-acetyl peak at δ 2.08 ppm. Detailed results regarding characterization of ChOS using ^{1}H-NMR and FTIR are summarized in Supplementary Table 1 and Supplementary Fig. 2.

Table 1
Characterization of ChOS.

Characterization of ChOS	
F_A (degree of acetylation)	$0.40_{(\text{determined by } ^1\text{H-NMR})}$
Ash content	8.3%
Solubility in H_2O	100%
Appearance	White powder (spray-dried)
Escherichia coli	Absent
Coliform bacteria	Absent
Salmonella spp.	Absent
Endotoxin	0.38 ± 0.13 EU mg^{-1}

Before use, ChOS were characterized regarding the distribution of the DP using HPLC (Fig. 1). The effect of endotoxin removal on the structure of the ChOS was determined with ^{1}H-NMR and revealed that the endotoxin cleansing procedure did not affect the ChOS structure, the degree of acetylation or the DP (Fig. 1, Supplementary Figs. 1 and 2, and Supplementary Table 1). The NMR and IR investigations showed that there was no change in ChOS materials subsequent to endotoxin removal (Supplementary Fig. 1 and Supplementary Table 1). All characteristic NMR and IR peaks were identical and the degree of acetylation was unaltered. Furthermore, the absence of changes in the intensity of the H-1 α peak, signaling the reducing end, and the HPLC elution pattern (Supplementary Fig. 2) also confirmed that the DP had not been affected by the endotoxin removal step.

3.2. Short-term effect of LPS and ChOS on human mesenchymal stem cells (hMSC)

We estimated proliferation rate and viability with an MTT proliferation assay and determined that 10 ng ml^{-1} LPS over a 7 day period did not affect the proliferation potential of hMSC (Supplementary Fig. 3).

During the analysis of cytokine secretion patterns, the presence of LPS is apparent after 3 days in culture (Fig. 2a and b), manifesting in a significant increase ($p < 0.05$) in the secretion of the inflammatory cytokines IL-6 and IL-8, as expected. RANTES and IL-12p(40), two cytokines not usually secreted under standard culturing conditions in hMSC, could also be detected with LPS present.

However, during the standard evaluation of proliferation, morphology and expression levels of genes previously shown to be sensitive to endotoxins, the effect of 10 ng ml^{-1} LPS could not be detected during the 7 day evaluation period of expansion (Fig. 2c and d). An increase in the expression of *TLR3* was observed between days 3 and 7 during the expansion of hMSC, but neither LPS nor ChOS affected its gene expression (Fig. 2c). The expression of *TLR4* did not change between 3 and 7 day periods of culture, and was likewise not significantly affected by the presence of LPS (Fig. 2c). The expression of *YKL-40* in the presence or absence of LPS and ChOS did not vary significantly, but a time-dependent increase was observed (Fig. 2d).

3.3. Quality of osteogenic differentiation in hMSC

The osteogenic differentiation potential of hMSC makes them an attractive tool to treat osteogenesis-related injuries, with or without the use of scaffolds and implants [20]. We therefore examined the expression of osteogenic marker genes in the presence of ChOS and LPS.

ChOS alone increased the expression of *COL1A2* ($p < 0.01$), whereas the presence of LPS completely abrogated this effect. No difference in the expression of *ALP* and *RUNX-2* was detected with ChOS. The expression of the osteogenic marker genes *ALP* ($p < 0.001$) and *RUNX-2* ($p < 0.01$) was significantly up-regulated with LPS.

Furthermore, we observed an increase in ALP activity ($p < 0.001$) and the deposition of calcium hydroxyapatite crystals in the presence of LPS or LPS in combination with ChOS, though no increase was detected when cells were differentiated with ChOS alone (Fig. 3b and c; Supplementary Fig. 4).

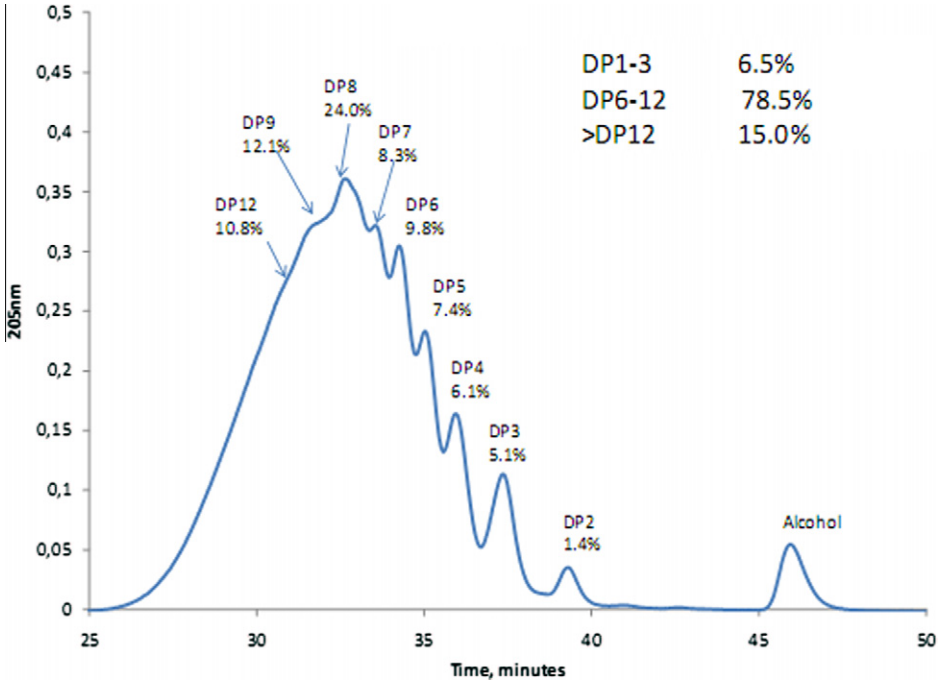

Fig. 1. HPLC characterization of ChOS. Distribution and quantity of different degree of polymerization oligomers was determined with HPLC using a TSK-Oligo column.

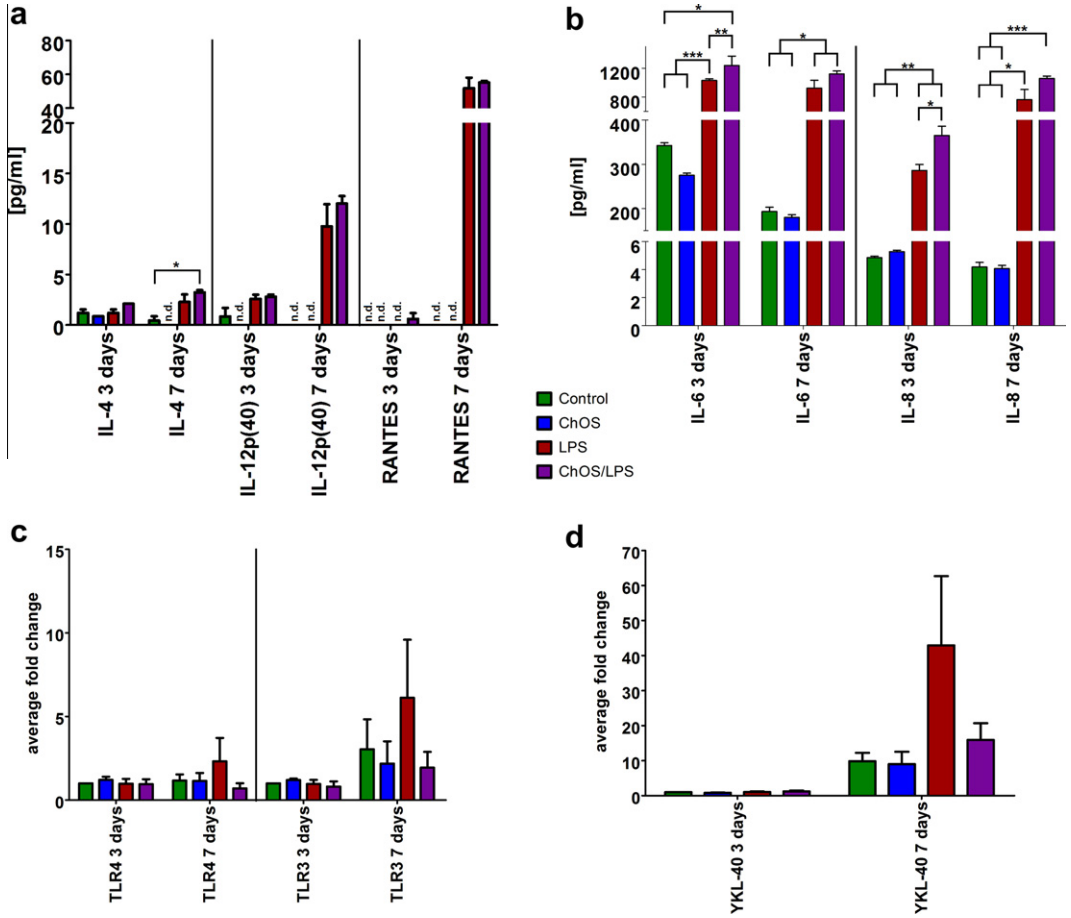

Fig. 2. Short-term effect of LPS and ChOS on hMSC. (a and b) Cytokine secretion of IL-4, IL-12p(40), RANTES, IL-6 and IL-8 after 3 and 7 days in expansion. A significant increase in IL-6 and IL-8 secretion was observed in the presence of endotoxins, as well as the induction of RANTES and IL-12p(40) secretion. Error bars are standard errors ($n = 2$) ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). (c and d) Expression of *YKL-40*, *TLR4* and *TLR 3* after 3 and 7 days of expansion. A time-dependent increase in expression can be observed for *YKL-40* independent of the presence of endotoxins. Error bars are standard errors ($n = 6$).

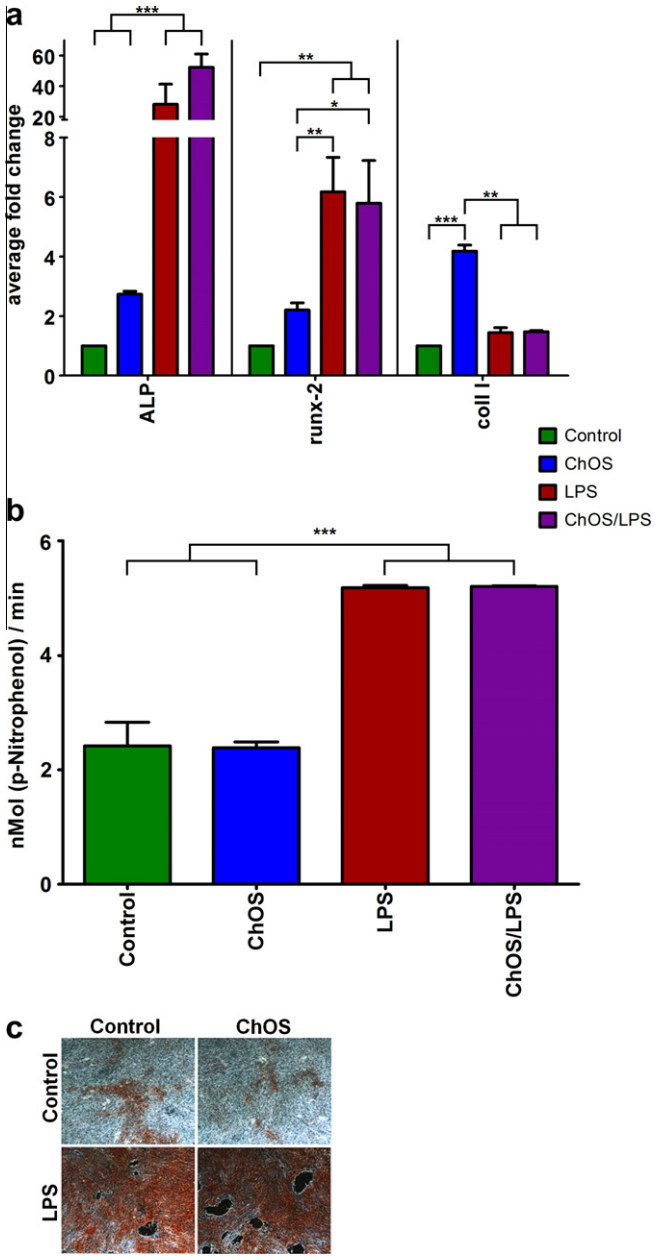

Fig. 3. Quality of osteogenic differentiation in hMSC. (a) Expression of alkaline phosphatase (*ALP*), runt-related transcription factor 2 (*RUNX-2*) and collagen type I (*COL1A2*) after 21 days of osteogenic differentiation. *ALP* and *RUNX-2* were significantly increased in the presence of endotoxins, whereas chitooligosaccharides alone but not in combination with LPS were able to significantly induce expression of *COL1A2*. Error bars are standard errors ($n = 6$) ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$); (b) alkaline phosphatase activity at 18 days during osteogenesis. Alkaline phosphatase activity was significantly induced in the presence of endotoxins. Error bars are standard errors ($n = 9$) ($^{***}p < 0.001$); c, Alizarin Red staining of calcification at 21 days of osteogenesis. Red staining is specific for calcium deposition (a representative picture of three donors).

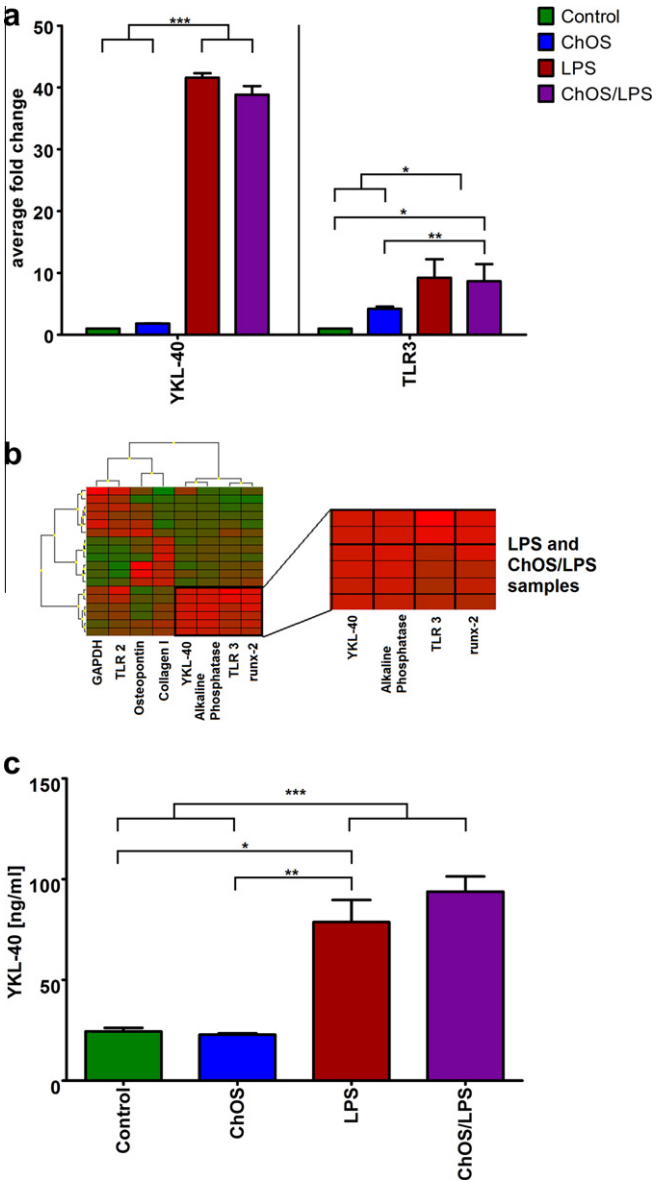

Fig. 4. Long-term effects of LPS and ChOS. (a) Expression of *YKL-40* and *TLR3*. Both genes were significantly increased in the presence of endotoxins. Error bars are standard errors ($n = 6$) ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$); (b) heatmap and hierarchical clustering of gene expression data autoscaled to genes. High expression of *YKL-40* is correlated with the expression of the osteogenic genes alkaline phosphatase (*ALP*) and *RUNX-2*, as well as Toll-like-receptor 3 (*TLR3*). A red color indicates high expression and green ndicates low expression ($n = 6$); (c) YKL-40 protein secretion during osteogenic differentiation of hMSC. Secretion of YKL-40 protein into medium supernatants was significantly increased in the presence of endotoxins. Error bars are standard errors ($n = 6$) ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

The increase in the expression of osteogenic marker genes is highly correlated with the increase in gene expression of *YKL-40* and *TLR3*, as demonstrated by the heatmap in Fig. 4b (also see Fig. 4a). Since *YKL-40* has been suggested to play a role in the remodeling of the extracellular matrix [12], the correlation of this gene with two osteogenic marker genes (*ALP* and *RUNX-2*) is not unexpected. The increase in *YKL-40* gene expression is furthermore consistent with increased secretion of YKL-40 protein into the cell culture medium (Fig. 4c).

3.4. Effect of LPS on cytokine secretion during osteogenesis

As observed during the expansion of hMSC, the presence of 10 ng ml^{-1} endotoxins during osteogenic differentiation cannot be conclusively determined from the quality of osteogenesis, i.e. gene expression, ALP activity and mineralization, but requires the analysis of the cytokine secretion profile. With 10 ng ml^{-1} LPS, the secretion of IL-4 ($p < 0.001$), IL-12p(40) ($p < 0.001$) and the pro-inflammatory cytokine TNF-α ($p < 0.001$) is up-regulated as compared to the control samples and ChOS alone (Fig. 5a). Again, RANTES was only induced in endotoxin-spiked samples. In

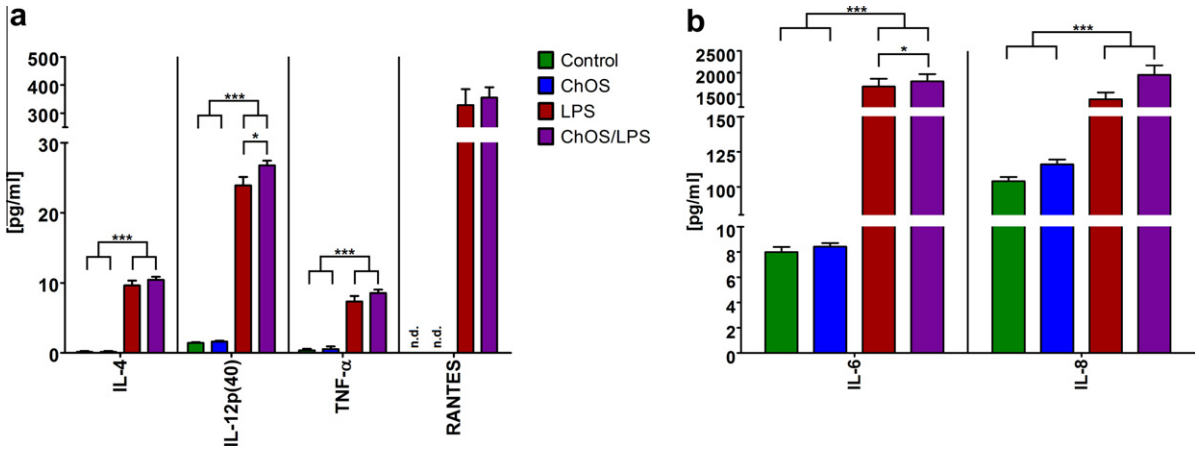

Fig. 5. Effect of endotoxins on cytokine secretion during osteogenesis. (a and b) Secretion of IL-4, IL-12p(40), TNF-a, RANTES, IL-6 and IL-8 at 20 days of osteogenic differentiation. Previously mentioned cytokines were significantly increased in the presence of endotoxins. RANTES secretion was only induced in samples containing LPS. Error bars are standard errors ($n = 6$) ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

addition, a substantial increase ($p < 0.001$) in the secretion of IL-6 and IL-8, corresponding to the results obtained during short-term culturing, was observed (Fig. 5b).

4. Discussion

Here, we report the effects of 10 ng ml^{-1} endotoxin contamination on the in vitro evaluation of the bioactivity of chitosan derivatives and how the presence of endotoxins can modify the results of in vitro bioactivity testing, in general leading to false-positive results. During standard evaluation of bioactivity, i.e. proliferation, morphology and gene expression of selected genes, the presence of endotoxins can escape notice unless cytokine secretion patterns are analyzed.

After 3 days in culture, the presence of LPS induced a significant increase in the secretion of inflammatory cytokines in hMSC. The activation of the innate immune system through interaction of ligands with TLRs induces a signaling cascade that results in the secretion of cytokines and chemokines, such as IL-6, IL-8, TNF-α and IFN-γ [21]. The increase in expression of IL-6 and IL-8 is an important part of the host defense in regulating inflammatory responses, and can directly affect bone metabolism [22]. The secretion of inflammatory cytokines can in turn activate the adaptive immune system, including the mobilization of antigen-presenting cells, the maturation of dendritic cells and the differentiation of naive T cells into activated T-helper 1 cells [23,24]. The activation of TLRs creates an inflammatory environment that stimulates cells of the adaptive immune system to migrate to the site of inflammation and remove the invading pathogens [23]. Mesenchymal stem cells (MSC) are known to secrete factors that can alter immune responses [25]. Several studies have shown that MSC suppress T- and B-cell activation, and inhibit the action of antigen-presenting cells as well as dendritic cell maturation [26]. TLRs 1–7 are expressed in MSC in vitro, and triggering of these receptors can alter migration and immunomodulatory functions of these cells [27].

Among the cytokines induced after challenge with LPS, IL-8 and TNF-α are known to be up-regulated in the T-helper 1 cell response, whereas IL-4 and IL-6 are associated with T-helper 2 cell responses [28]. The proinflammatory cytokines TNF-α, IL-6 and IL-8 are expected to decrease wound healing by increasing cellular activation and chemotaxis [28]. IL-12p(40) is a potent regulator of cell-mediated immune responses and activates natural killer cells and T cells [29]. RANTES is known as a chemoattractant to monocytes, memory T cells and basophils, and has been shown to be increased in patients suffering from sepsis. This cytokine was identified as a key player in the uncontrolled expression of proinflammatory cytokines during inflammation, which is further supported by the notion that RANTES is rarely expressed in normal adult tissues but can be found at sites of inflammation [29–31].

TLRs were established as important parts of innate immunity, providing a link between innate and adaptive immune system activation [21]. The conserved structure of these receptors aids in the pattern recognition of conserved motifs in the make-up of invading microorganisms, and TLRs have been linked to epithelial homeostasis by inducing proliferation and tissue repair after injury [25]. We determined the effect of LPS on the expression of its direct receptor counterpart TLR4 and the closely related receptor TLR3, which is believed to be important in facilitating stress responses in hMSC [27]. TLR3 not only reacts to its known ligand, poly(I:C), but can also respond to ligands that bind to closely related TLRs [11]. In the present study, small amounts of LPS did not affect the expression levels of genes in hMSC previously shown to be sensitive to the presence of endotoxins.

With the aid of a heatmap based on hierarchical clustering, the correlation between gene expression and the presence of LPS was visualized [32]. The increase in the expression of the osteogenic marker genes *ALP* and *RUNX-2* is highly correlated to the gene expression levels of *YKL-40* and *TLR3*. *RUNX-2* is one of the key players involved in osteogenic differentiation [33]. It can be detected throughout osteogenic and chondrogenic differentiation, and is essential for the expression of downstream transcription factors [34]. Even though *RUNX-2* is not specific for osteogenic differentiation, it maintains a pool of undifferentiated osteoprogenitor cells and induces the expression of osteogenesis-related genes like *OPN* (osteopontin), *OCN* (osteocalcin) and *COL1A2* [35,36]. An early event during osteogenic differentiation of hMSC is the reduction of proliferative abilities, accompanied by increased expression of the early marker gene *ALP* (alkaline phosphatase) [37].

A large subset of signaling molecules and receptors expressed during bone turnover are shared with the immune system [38]. This is caused, in part, by the fact that osteoclasts, the bone resorbing cells, and immune cells are both derived from the hematopoietic lineage [39]. Osteoclasts can react to cytokines produced by macrophages and other immune cells during inflammation, and can activate bone remodeling pathways in vivo [40].

The expression of the osteogenic marker genes *ALP* and *RUNX-2* was not affected by ChOS alone, whereas the expression levels of *COL1A2* were significantly increased. The presence of LPS completely abrogated this effect on *COL1A2* gene expression. The up-regulation of *COL1A2* expression in the presence of ChOS alone might be explained by the heparin binding site found in the collagen type I protein. Heparin is a glycosaminoglycan with a variable

structure, but one of the typical building blocks is the amino sugar D-N-acetylglucosamine [41]. This amino sugar comprises approximately 40% of the ChOS mixture used in this study and might be able to interact with the heparin binding site on the collagen type I protein. This effect is not observed when ChOS is used in combination with LPS. One possible explanation for this might lie in the direct interaction between the two compounds. Chitosan was shown to form stable complexes with LPS, based on the formation of hydrogen bonds and electrostatic interactions [7]. Water-soluble derivatives with a high degree of deacetylation, such as the ChOS used in this study, are expected to interact with the lipid A moiety of endotoxins and bind with high affinity [7]. A high degree of interaction of the chitosan derivative with endotoxins could sterically hinder possible binding sites of ChOS in the cell and might therefore be responsible for the abrogation of increased *COL1A2* expression. However, the effect of the endotoxin itself on cellular functions should not be affected by this interaction. Even under optimal interaction conditions, approximately 30% of the endotoxin will be unbound and able to interact with cellular receptors [42].

Chitosan is considered to be a promising candidate for applications in regenerative medicine and tissue engineering, partly due to recent reports on the positive influence of chitosan on osteogenesis in vitro and in vivo [3,4]. Lahiji et al. [43] showed that human osteoblasts grown on chitosan membranes with a 90% degree of deacetylation sustained a spherical morphology as compared to spindle-shaped cells on tissue culture plastic, and preserved collagen type I expression during short-term culture. Similarly, Amaral et al. [5] demonstrated that chitosan membranes could promote the differentiation of osteoprogenitor cells and aid in bone formation. Neither study reported endotoxin testing of the chitosan starting material and both focused on the use of chitosan as a biological growth substrate for cells in the form of membranes and bioactive coatings. ChOS, as used in the present study, are low-molecular-weight derivatives of chitosan that has undergone regioselective enzymatic degradation. Since the biological potential strongly depends on chemical properties, differences in bioactivity between chitosan and ChOS can be assumed [44]. Additionally, we have recently shown that pure, endotoxin-free hexamer fractions of chitosan and chitin oligomers increase the expression of *TLR3*, *RUNX-2* and *COL1A2* during osteogenic differentiation of hMSC, but do not affect the mineralization process [45]. Mass production of pure oligomer fractions with a well-defined degree of polymerization is laborious and expensive, which is why many studies focus on the use of ChOS mixtures to evaluate bioactivity [46,47]. This introduces a higher level of complexity despite advanced analysis methods, since both active and inhibitory oligomer fractions might be present simultaneously, affecting the biological activity [48].

The presence of LPS resulted in increased osteogenic marker gene expression, elevated ALP activity and increased deposition of calcium hydroxyapatite crystals. Improvement of osteogenic differentiation in MSC in the presence of LPS has been described in several studies and was shown to be coupled with increased activation of the ERK pathway and dependent on the duration of endotoxin exposure [49,50]. The main event connecting the presence of endotoxins to bone biology is the induction of proinflammatory cytokines such as IL-6 after ligation with TLR4. IL-6 was tightly linked to osteoclast-induced bone resorption [22,51]. This also explains why an increase in osteogenic differentiation of hMSC can be observed in vitro, whereas increased rates of bone-turnover are induced in vivo or in co-culture with preosteoclasts.

5. Conclusion

We have shown that the presence of endotoxins in chitosan derivatives can profoundly alter the performance of a potential biomaterial in vitro. Endotoxin-contaminated ChOS will improve osteogenic differentiation and deposition of calcium hydroxyapatite crystals without affecting cell viability of hMSC. This positive effect on osteogenesis is abrogated after appropriate endotoxin removal.

Endotoxin contamination is difficult to avoid completely during production and handling of natural substances. With the proper testing and handling of starting materials derived from natural materials and the evaluation of cytokine secretion patterns, such materials can be useful tools in biomaterials research. Spiking samples with known amounts of endotoxins can be beneficial, as well as testing bioactivity in the presence of endotoxin inhibitors such as polymyxin B [52]. The inclusion of simple quality control procedures to standard evaluation protocols of biomaterials will reduce the potential discrepancies between in vitro and in vivo studies, where a formerly bioactive and successful biomaterial has reduced bioactivity because of the strict rules for material safety in clinical practice.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1–5, are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi: http://dx.doi.org/10.1016/j.actbio.2012.08.043.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2012.08.043.

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