applied sciences

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The Synergic Effect of Terpenoid and Steroidal Saponins Can Improve Bone Healing, by Promoting the Osteogenic Commitment of Adipose Mesenchymal Stem Cells: An In Vitro Study

Gloria Bellin [1,2], Chiara Gardin [1,2], Letizia Ferroni [1,2], Paolo Ghensi [3,4], Barbara Zavan [1,2,*,†] and Marco Tatullo [5,*,†]

1 Maria Cecilia Hospital, GVM Care & Research, via Corriera 1, 48033 Cotignola, Ravenna, Italy
2 Department of Medical Sciences, University of Ferrara, Via Fossato di Mortara, 70, 44121 Ferrara, Italy
3 Centre for Integrative Biology (CIBIO), University of Trento, Via Sommarive, 9, 38123 Povo, Trento, Italy
4 Department of Neurosciences, University of Padova, Via Giustiniani, 5, 35128 Padova, Italy
5 Marelli Health, Tecnologica Research Institute, Stem cell Unit, 88900 Crotone, Italy
* Correspondence: barbara.zavan@unife.it (B.Z.); marco.tatullo@tecnologicasrl.com (M.T.)
† co-last authors.

Received: 26 July 2019; Accepted: 15 August 2019; Published: 20 August 2019

Featured Application: Applied phytochemistry can improve the translational tissue engineering, by selective triggering of specific biological processes, ensuring a low impact on cell viability and the overall biocompatibility. Better knowledge of the synergic action of saponins, and the determination of the most effective concentrations, can be used in the production of biomedical scaffolds for critical-sized bone defects, and for long-term bone repairs in such patients with chronic degenerative processes of bone tissue.

Abstract: Bone regeneration involves several biological processes that consistently impact the quality of tissue healing. An important step consists of the local recruitment and differentiation of mesenchymal stem cells that migrate in the site to regenerate from bone marrow. Mesenchymal stem cells (MSCs) may be pushed towards osteogenic commitment by specific substances, often naturally present in plants. Yunnan Baiyao (YB) is a Chinese herbal medicine, mainly working through the synergic effect of terpenoid and steroidal saponins. YB is well known for its numerous biomedical effects, including the ability to favor improved bone tissue healing. In our in vitro study, we used adipose mesenchymal stem cells (ADSCs) as a study-model: We selected samples to harvest and isolate ADSCs and investigate their viability; moreover, we performed bone-related gene expression to evaluate the differentiation of MSCs. To confirm this behavior, we analyzed alkaline phosphate activity and calcium deposition, with ADSCs cultured in basal and osteogenic media, with YB at different concentrations in the medium, and at different time-points: 7, 14 and 21 days. Our results indicate that the synergic effect of terpenoid and steroidal saponins slightly favor the late ADSCs differentiation towards the osteoblasts phenotype. In osteogenic committed cells, the treatment with the lower dose of YB promoted the up-regulation of the alkaline phosphatase gene (ALPL) at day seven and 14 ($p < 0.01$); at day 21, the alkaline phosphatase (ALP) activity showed a slight increase, although in basal condition it maintains low rates. We assume that such molecular synergy can promote the osteogenic commitment of adipose mesenchymal stem cells, thus improving the timing and the quality of bone healing.

Keywords: bone repair; mesenchymal stem cell; adipose mesenchymal stem cell; osteogenic differentiation

1. Introduction

Bone is a biological tissue able to regenerate in case of traumas or degenerative pathologies. After bone injuries, the physiopathology of the tissue healing follows different stages. The first key-step is the chemotaxis of immune cells from the bloodstream, as they will promote the activation of the inflammatory response to the injury [1]. At the same time, resident and "sleeping" mesenchymal stem cells (MSCs) activate and migrate into the injured tissue, from the bone marrow. MSCs effectively start the repairing process [2]. Locally, MSCs will differentiate into chondrocytes and osteoblasts. The healing process continues with the remodeling of the newly formed bone, so restoring the anatomical shape and function [3].

In healthy people, about 10% of fracture healings may go wrong [4]; on the other hand, the risk of experience a problem in healing is increased 6- to 7-fold for type 1 diabetes patients, and 1.4- to 1.7-fold for people with type 2 diabetes [5]. Moreover, the healing time is typically prolonged in about 87% of diabetics, and the risk of complication, such as delayed union is 3.4-fold higher [6–8]. Besides, several studies have reported that diabetic patients have an impairment of the MSC osteogenic commitment and osteoblasts function: Both these conditions certainly contribute to worsening the bone healing [9]. It this landscape, the bone healing and repairing may need to be improved with biocompatible and osteoinductive therapies to promote bone regeneration.

The Yunnan Baiyao (YB) is a well known Chinese herbal medicine, firstly introduced in the healthcare market in 1914 [10]. The main compounds of YB are: Radix Notoginseng (Panax pseudoginseng var. notoginseng (Burkill) G. Hoo & C.L. Tseng), Borneolum Synthcticum (Cinnamomum camphora (L.) J. Presl), and Radix Aconiti Kusnezoffii (Aconitum Kusnezoffii Reichb.); however, the exact composition is confidential, because it is one of the two Class-1 protected traditional Chinese Medicines [10,11]. Nevertheless, some studies tried to characterize the main active principles working in YB, revealing the presence of saponins, terpenoids and steroid glycosides [12,13]. Recently, Liu et al. [11] reported the presence of five terpenoid saponins and eight steroidal saponins in YB powder and capsules that contribute to its therapeutic efficacy. In fact, YB has been reported to have several therapeutic properties in clinical cases reporting severe inflammation or tissue damage.

Interestingly, the most reported pharmacological effects of YB are related to its hemostatic properties [14]. The anti-inflammatory effect, the ability to improve wound healing, and bone regeneration have also recently been added to this [10,11]. Despite all these effective properties, there are also limitations mainly related to the scarce information reported in the scientific literature. YB effects have not been well investigated in vitro, and most of the studies on YB are published in Chinese literature, making them difficult to be understood globally [15,16]. Specifically, the effects of YB on bone regeneration are reported in few papers, and they describe contrasting effects. Ou et al. [17] reported that YB could improve the fractures healing in a rabbit model; on the contrary, Zhang et al. [18] indicated that YB does not increase the formation of new bone, after a third molar extraction in humans.

We aim to give a robust and reliable point of view, taking into consideration the previous literature, about the effects of YB on bone regeneration. In more detail, we will investigate whether YB can induce new bone formation through the differentiation of MSCs towards osteogenic phenotype. Our null hypothesis emphasizes the synergic effect of terpenoid and steroidal saponins on the overall process of bone healing: we assume that such molecular synergy will promote the osteogenic commitment of adipose mesenchymal stem cells, thus improving the timing and the quality of bone healing.

2. Materials and Methods

2.1. Isolation of Mesenchymal Stem Cells from Adipose Tissue (ADSCs)

Samples of adipose tissue have been obtained from abdominoplasty of three healthy patients. Written consent has been required, in agreement with the guidelines of Clinic of Plastic Surgery, working within the Padua University Hospital. The collected samples were washed twice with phosphate-buffered saline (PBS, EuroClone, Milan, Italy) and cut down in 1 cm^2 pieces. They were

digested with 0.075% collagenase from *Clostridium hystoliticum* Type II (Sigma-Aldrich, St. Louis, MI, USA) in Hank's Balanced Salt Solution (HBSS, Lonza, Basel, Switzerland) and placed under stirring for 3 h at room temperature (RT). When the digestion was completed, collagenase activity was quenched with an equal amount of basal medium, made with Dulbecco's modified Eagle's medium (DMEM, Lonza) added with 10% fetal bovine serum (FBS, EuroClone) and 1% Penicillin/Streptomycin (P/S, Lonza). After the removal of non-digested material, by means of a careful filtration, the eluate was centrifugated at 12,000 rpm for 4 min. The obtained mesenchymal stem cells from adipose tissue (ADSCs) were seeded in plates with basal medium and incubated at 5% CO_2 at 37 °C. Culture medium was changed every two days (d).

2.2. Cell Culture

Mesenchymal stem cells from adipose tissue (ADSCs) were expanded and cultured in basal medium (BM) or osteogenic differentiating medium (ODM) (DMEM, 10% FBS, 1% P/S, 10 ng/mL basic fibroblast growth factor (bFGF, ProSec), 10 mM β-glycerophosphate (Sigma-Aldrich), 10 nM dexamethasone (Seromed, Munich, Germany) up to the day 21.

After that, 50 mg of YB powder was solubilized in 1% DMSO, in sterile water, and ADSCs were cultured with 25, 50, 100 or 200 µg/mL of YB. Experiments were performed with three different cell preparations and repeated three times each.

2.3. Methyl Thiazolyl-Tetrazolium (MTT) Assay

Cell viability was investigated through a methyl thiazolyl-tetrazolium (MTT) assay, according to the protocols previously described [19]. Briefly, 1×10^4 cells at p5 were seeded onto 48-well plates and treated as previously described (Section 2.2). After 1, 3, 5, 7, 14 or 21 days from seeding, the culturing medium was removed, and cells were incubated for 3 h at 37 °C in 0.5 mL of 0.5 mg/mL MTT solution. After removal of the MTT solution, 0.5 mL of 10% dimethyl-sulfoxide (DMSO) in isopropanol was added for 15 min at RT. Absorbance (A) values at 570 nm were recorded in duplicate, using a multilabel plate reader (Victor 3, Perkin Elmer, Milan, Italy).

2.4. Real-Time PCR Assay

The total RNA was extracted from each cell culture, by using the Total RNA Purification Plus Kit (Norgen Biotek Corp., Thorold, Canada), including DNase Digestion. The RNA quality and concentration were measured with NanoDrop™ ND-1000 (Thermo Scientific, Waltham, MA USA); 500 ng of total RNA for each sample were reverse transcribed, by using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and following the manufacturer's protocol. Human primers were selected for each target gene with Primer 3 software (Table 1). Real-time PCRs were carried out using the designed primers at the concentration of 300 nM and FastStart SYBR Green Master (Roche Diagnostics, Penzberg, Germany) on a Rotor-Gene 3000 (Corbett Research, Cambridge, UK). Thermal cycling conditions were as follows: 15 min of denaturation at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C; annealing for 30 s at 60 °C, and elongation for 20 s at 72 °C. Afterward, MSCs cultured in BM and in ODM were both analyzed, as two different groups: for each group, the samples not-treated with YB were used as control. The obtained values were normalized with the expression of the transferrin receptor (TFRC), as per internal reference: it did not change under our experimental conditions.

Table 1. Human primer sequences. Runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALPL), osteocalcin (OC), and transferrin receptor (TFRC).

Gene	Sequences (5′–3′)	Amplicon (pb)
RUNX2	AGCCTTACCAAACAACACAACAG CCATATGTCCTCTCAGCTCAGC	175

Table 1. *Cont.*

Gene	Sequences (5′–3′)	Amplicon (pb)
ALPL	GGCTTCTTCTTGCTGGTGGA CAAATGTGAAGACGTGGGAATGG	181
OC	GCAGCGAGGTAGTGAAGAGAC AGCAGAGCGACACCCTA	193
TFRC	TGTTTGTCATAGGGCAGTTGGAA ACACCCGAACCAGGAATCTC	222

2.5. Alkaline Phosphatase Assay

The intracellular alkaline phosphatase (ALP) activity was detected, by means of the colorimetric Alkaline Phosphatase Assay Kit (Abcam, Cambridge, UK). After 7, 14 and 21 days of culture, cells were washed with PBS, homogenized with ALP Assay Buffer, and centrifuged at 13,000 rpm for 3 min. A standard curve was drawn, using the corrected absorbance values of standards.

2.6. Alizarin Red S Staining Assay

Alizarin Red S staining was used to detect extracellular mineral deposits. Cells were fixed and stained with 40 mM Alizarin Res-S solution (pH 4.2) for 20 min at RT. Afterward, cells were washed with ddH_2O and photographed by an optical microscope. The staining that remains after several washes was extracted with 10% cetylpyridinium chloride (CPC) solution and absorbance values at 570 nm were recorded in duplicate, using a multilabel plate reader.

3. Results

3.1. ADSCs Viability with Different YB Concentrations

The cytotoxic effects of different YB concentrations have been investigated (Figure 1). YB has been added, at different dosages, to ADSCs culture medium. Moreover, the same assay has been used to assess if YB can impact the osteogenic commitment of ADSCs.

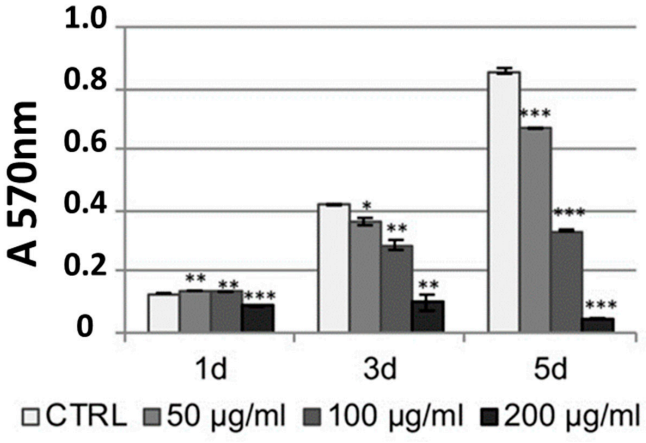

Figure 1. Adipose mesenchymal stem cells (ADSCs) viability measured by a methyl thiazolyl-tetrazolium (MTT) assay. Viability of control ADSCs and ADSCs treated with 50, 100 or 200 µg/mL of Yunnan Baiyao (YB) at 1, 3 and 5 day of culturing in basal medium. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control (CTRL).

The experimental sessions investigated the ADSCs viability under different culturing conditions. ADSCs viability increased over 21 days, when they were cultured both in BM (Figure 2a) and in ODM (Figure 2b), showing a higher rate in ODM.

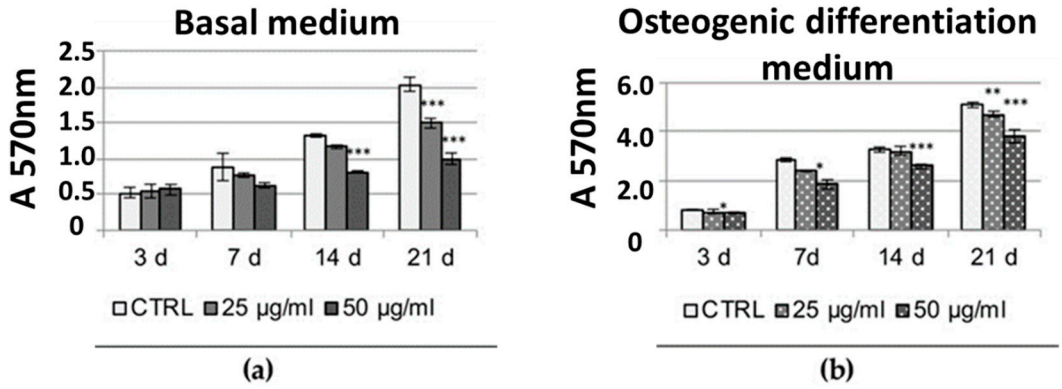

Figure 2. ADSCs viability measured by MTT assay. (**a**) Viability of control ADSCs and ADSCs treated with 25 µg/mL or 50 µg/mL of YB at 3, 7, 14 and 21 day of culturing in basal medium. (**b**) Viability of control ADSCs and ADSCs treated with 25 µg/mL or 50 µg/mL of YB at 3, 7, 14 and 21 day of culturing in osteogenic differentiation medium. Absorbance was measured at 570 nm. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. CTRL.

The addition of different YB aliquots in cell culture medium led to decreasing cell viability. MTT analysis revealed that YB aliquots over 100 µg/mL severely reduced the overall cell viability (Figure 1). After having reduced the YB aliquot to 50 µg/mL, the lower dose of YB did not modulate ADSCs viability until day 14 ($p > 0.05$), while at day 21 the reduction became significant in both culturing conditions ($p > 0.01$). In culturing condition with 50 µg/mL of YB, the viability rate was lower than the control at 14 and 21 days ($p < 0.001$).

3.2. Impact of YB on Osteogenic Gene Expression

Runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALPL) and osteocalcin (OC) gene expression was measured by real-time PCR, to assess the effect of two different doses of YB on ADSCs ability to differentiate towards osteogenic lineage. Our results showed that cells maintained in ODM had a higher expression of the osteogenic markers, compared to those cultured in BM (Figure 3).

Analyzing, in depth, the effects of YB on cells cultured in basal conditions, we observed that Runx2 expression was downregulated at 7 days, with respect to control ($p < 0.05$); on the other hand, after 14 days, Runx2 expression was similar in both the culturing conditions. The overcoming of Runx2 expression, with respect to the control, was reached after 21 days of treatment, with both of the YB concentrations ($p < 0.05$) (Figure 3a).

In the same basal conditions, ALPL gene expression was downregulated when cells were treated with YB at 50 µg/mL, both after 14 and 21 days ($p < 0.05$). Conversely, YB at 25 µg/mL did not modify ALPL expression at all time points ($p > 0.05$) (Figure 3b).

The late osteogenic gene OC followed a different trend. At day 7, it was equally expressed by control and treated ADSCs; on the other hand, at day 14 and day 21, the treatment with YB at 25 µg/mL significantly up-regulates OC expression ($p < 0.05$) and the treatment with YB at 50 µg/mL significantly down-regulated OC expression ($p < 0.05$) (Figure 3c).

The results were different for ADSCs cultured in ODM. The analysis of Runx2 gene expression showed that at day 7 and 21 there were no differences between the culturing conditions ($p > 0.05$); at day 14, the 25 µg/mL YB-treated cells expressed more Runx2, compared to the control ($p < 0.01$) and the 50 µg/mL YB-treated cells expressed less Runx2, compared to the control ($p < 0.01$) (Figure 3d).

In osteogenic committed cells, the treatment with the lower dose of YB promoted the up-regulation of ALPL gene at day 7 and 14 ($p < 0.01$); at day 21, the ALPL expression was down-regulated ($p < 0.01$) (Figure 3e).

OC expression followed the same trend in all time points. Culturing conditions with YB at 25 µg/mL increased OC expression ($p < 0.05$); YB at 50 µg/mL decreased OC expression, with respect to the control ($p < 0.01$) (Figure 3f).

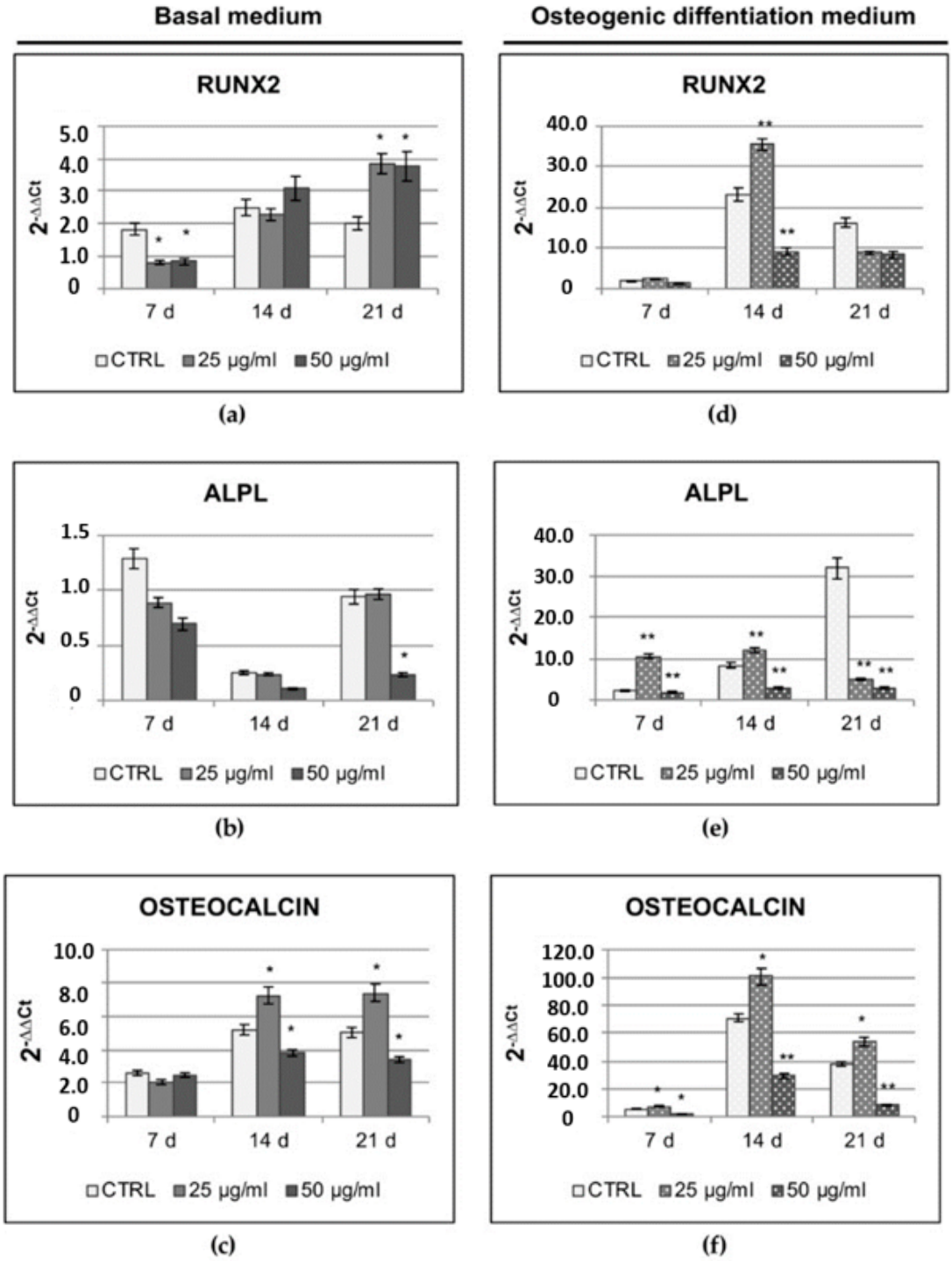

Figure 3. Gene expression of osteogenic markers. (**a**–**c**) Gene expression of Runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALPL) and osteocalcin (OC) in control ADSCs and ADSCs treated with 25 µg/mL or 50 µg/mL of YB at 3, 7, 14 and 21 day of culturing in basal medium. (**d**–**f**) Gene expression of Runx2, ALPL and OC in control ADSCs and ADSCs treated with 25 µg/mL or 50 µg/mL of YB at 3, 7, 14 and 21 day of culturing in osteogenic differentiation medium. * $p < 0.05$; ** $p < 0.01$ vs. CTRL.

3.3. Impact of YB on Alkaline Phosphatase Activity

Intracellular ALP activity (expressed as U/mL) was measured on ADSCs in both BM and ODM conditions, after 7, 14 and 21 days of cell culture. ALP activity resulted in increased cells cultured in ODM, compared to cells grown in BM (Figure 4). The treatment with YB at 25 µg/mL did not induce any variation in ALP activity, both in BM and ODM at all time-points ($p > 0.05$). On the contrary, the treatment with YB at 50 µg/mL induced a decreased ALP activity, in both culturing conditions on day 7 and, more evident, at day 14 ($p < 0.05$). At day 21, the ALP activity showed a slight increase, although in basal conditions it maintains low rates ($p < 0.01$).

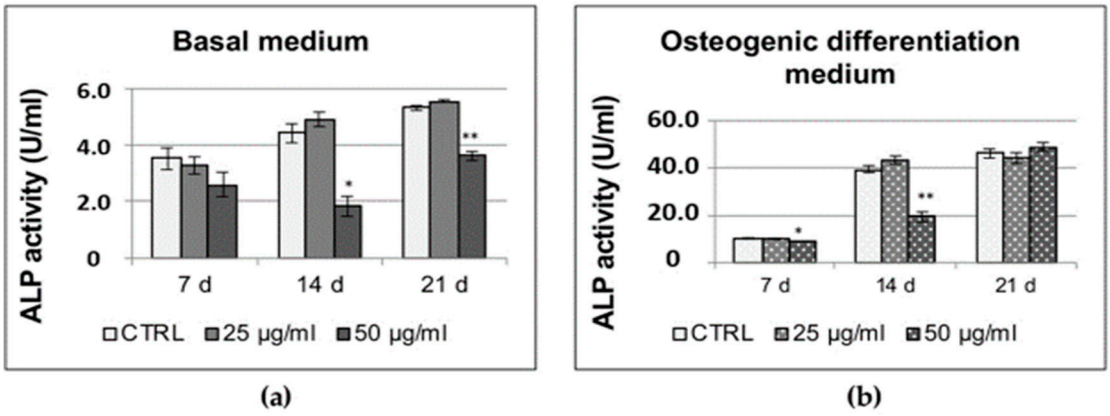

Figure 4. Alkaline phosphatase (ALP) activity assay. (**a**) ALP activity of control ADSCs and ADSCs treated with 25 µg/mL or 50 µg/mL of YB at 3, 7, 14 and 21 day of culturing in basal medium. (**b**) ALP activity of control ADSCs and ADSCs treated with 25 µg/mL or 50 µg/mL of YB at 3, 7, 14 and 21 day of culturing in osteogenic differentiation medium. * $p < 0.05$; ** $p < 0.01$ vs. CTRL.

3.4. Impact of YB on Alizarin Red S Staining

The impact of YB on osteogenic commitment showed by ADSCs was assessed by an Alizarin Red S staining assay. In basal medium, no staining was evident at day 7 and 14. On the contrary, initial staining was recorded at day 21, slightly reduced in YB-treated ADSCs ($p < 0.01$) (Figure 5a,b). ADSCs cultured in ODM showed mineral deposits at day 21; the staining quantification highlighted higher values in cells treated with YB at 25 µg/mL, after 7 and 21 days ($p < 0.05$); otherwise, the higher dose of YB (50 µg/mL) seemed to decrease the number of extracellular mineral deposits at day 7 ($p < 0.001$) (Figure 5c,d).

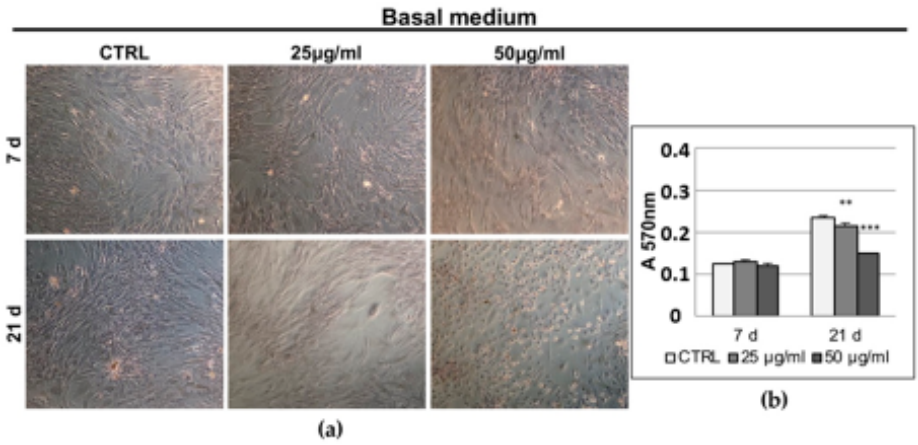

Figure 5. *Cont.*

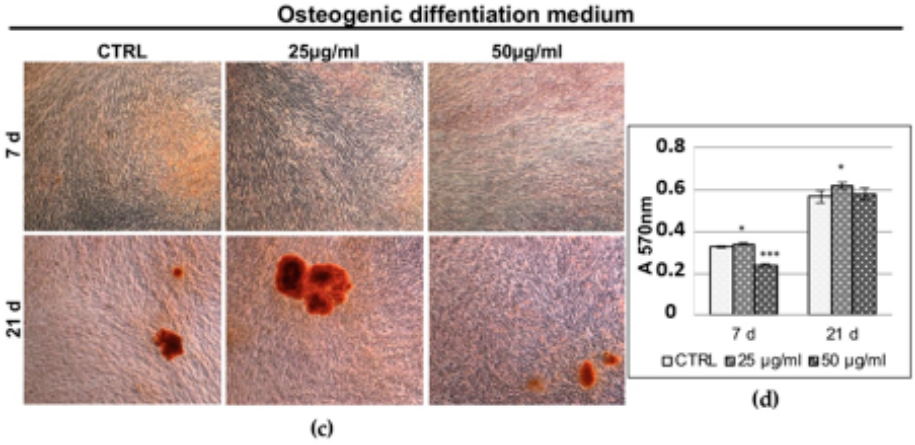

Figure 5. Alizarin red S (ARS) stainingassay. (**a**) ARS assay in control ADSCs and ADSCs treated with 25 µg/mL or 50 µg/mL of YB at 7 and 21 day of culturing in basal medium. Magnification 20X. (**b**) Quantification of ARS assay in control ADSCs and ADSCs treated with 25 µg/mL or 50 µg/mL of YB at 7 and 21 day of culturing in basal medium. (**c**) ARS assay in control ADSCs and ADSCs treated with 25 µg/mL or 50 µg/mL of YB at 7 and 21 day of culturing in osteogenic differentiation medium. Magnification 20X. (**d**) Quantification of ARS assay in control ADSCs and ADSCs treated with 25 µg/mL or 50 µg/mL of YB at 7 and 21 day of culturing in osteogenic differentiation medium. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. CTRL.

4. Discussion

MSCs differentiation is a fundamental step toward bone repair. The Yunnan Baiyao (YB), a Chinese herbal medicine, is well-known for its beneficial effects, especially on bone tissue healing. YB has been reported to have several biological effects, such as hemostatic properties; effective hemostasis after bone trauma is an important step to achieve good healing. Currently, surgeons have the challenge to choose a hemostatic material to ensure an efficient and immediate reduction of hemorrhagic phenomena in the surgical site. A good hemostasis followed by a strong anti-inflammatory effect ensures the proper biological environment to emphasize the osteogenic properties, here investigated, of YB.

In our article, we decided to investigate the effects of YB on MSCs osteogenic differentiation, so as to better understand its potential applications in bone regeneration.

Our MTT assay highlighted that YB administered at doses higher than 50 µg/mL, severely affects the cell viability. These results are in accordance with the study of Wirth et al., although they worked on other cell types [14]. Starting from this information, we established 50 µg/mL as the highest dose of YB to be safely administered.

We analyzed the gene expression of three osteogenic markers: Runx2, ALPL, and OC. Runx2 is an early osteogenic marker: It is considered essential for the osteogenic differentiation, because it promotes the expression of osteoblastic genes, such as osteocalcin [20]. Moreover, it was demonstrated that Runx2 improves the ADSCs differentiation towards osteoblasts, by increasing the ALP expression in addition to OC expression as well [21]. ALP is also considered an early marker of osteogenic differentiation and it is assumed that its function is to promote matrix mineralization [22]. Several studies demonstrated that ALP gene expression increases in the middle of the osteoinduction process, and then quickly decrease at the end of the osteoinduction process, followed by an intense deposition of bony matrix [23–25].

OC is a major protein of the bone extracellular matrix and it is expressed exclusively by mature osteoblasts. OC plays a pivotal role in the production of hydroxyapatite crystals [26,27]. Furthermore, it is considered an indicator of osteoblast differentiation. After a careful analysis of the gene expression carried out from our study, the substantial increase in Runx2, ALPL and OC expression observed in ODM cultured ADSCs indicates that ADSCs were differentiating into osteoblasts; the addition of 25 µg/mL of YB enhanced the overall expression of these markers, triggering osteogenesis. On the

contrary, the treatment with 50 µg/mL of YB reduced the MSCs differentiation, decreasing the expression of all the osteogenic genes at each time point.

Recent studies highlighted a reduced expression of Runx-2 in MSCs from type 2 diabetes patients, correlated to a decreased ability of MSCs to naively differentiate into osteoblasts [9,28,29].

Furthermore, new bone formation in diabetic patients is typically reduced by an impairment of osteoblast activity, associated with a reduction of OC production, while the activity of the osteoclasts remains unchanged [30,31].

Our study suggests that YB could be helpful to accelerate fracture healing, as it is able to promote Runx2, ALP and OC expression, when MSCs are cultured in ODM. Interestingly, the upregulation of OC in cells cultured in basal medium, suggests that also in the presence of a not favorable environment, just like the diabetic tissues, lower doses of YB can make a small but significant contribution to improve bone regeneration.

Several studies have demonstrated that MSCs increase the production of ALP only in the presence of osteogenic stimuli [24,27,32]. In agreement with these studies, our results showed a huge production of ALP, only in the presence of an osteogenic medium. Contrarily, a lower dose of YB did not show improved ALP activity, compared to the control. However, the Alizarin Red S staining assay highlighted that YB administered at 25 µg/mL promotes calcium deposition in the short term and long term.

A recent study investigates the odonto/osteogenic induction capacity of YB on stem cells from apical papilla [32,33]. Pang and colleagues reported that YB powder is rich in calcium ions, and it is the main reason for its well-known hemostatic properties. In fact, calcium ions can stimulate the local production of hemostatic agents; moreover, the calcium ions interact with cytokines and growth factors, so stimulating the local bone formation [32–35]. Mesenchymal stem cells (MSCs) are located in several areas of the human body. Some of them are located in strategic points, in a "sleeping" state, waiting for a stimulus able to re-activate them. In the bone marrow, MSCs are richly present, as bone trauma frequently requires a large activation of regenerative processes, to replace the anatomy and the functionality of the area injured by the pathological process. MSCs are also present in strategic tissues of the human organism, such as the oral cavity: These MSCs are often called oral-derived MSCs (OMSCs). OMSCs have been reported to have a remarkable proliferative ability, this can be truly important in such degenerative processes developing on periodontal tissues that can lead to loss of teeth.

Interestingly, as a result of this research, it has emerged that YB promotes cell differentiation through the activation of the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathway [35–38]. However, several differences have been pointed out between that study and our study. Firstly, a different source for MSCs was used; moreover, the YB preparation was highly different, and the time-points we used were longer than the other study. Probably, this is the reason why in our study a 50 µg/mL concentration of YB did not stimulate cell differentiation, while Pang and colleagues affirmed that it is the best concentration to be used for the experimental sessions on bone healing. In the current literature, no preliminary information has been reported about the use of saponins from YB in bone tissue engineering; nevertheless, it is important to take into consideration this strategic application in the treatment of bone damage [36–38]. An important field of application of bone regeneration is the dental sciences: In fact, there are several clinical conditions in dentistry requiring proper and timely bone healing, such as periodontal diseases [39], and functional and anatomical rehabilitation after maxillofacial surgery [40,41]. In this landscape, the authors suggest that future impacting researches should be performed by the scientific community. In more detail, the researchers should investigate on the main translational effects of YB in bone regeneration, as it could have a positive impact on the main regenerative protocols as well as in specific clinical conditions, such as syndromic conditions [42–44] or oncological conditions [45–47], or any pathological condition [48–50] where drugs should be carefully limited.

5. Conclusions

Our results overall indicate that YB may favor stem cell differentiation, the especially when they are cultured in an osteogenic medium. The assays on osteogenic commitment confirmed that the highest YB concentration does not promote ADSCs osteogenic differentiation; on the contrary, it may be highly cytotoxic. Our results also indicate that the effects of YB on ADSCs differentiation are dose-dependent, and they are closely related to the presence of an osteogenic medium. The ADSCs cultured in a classic medium are not stimulated to differentiate towards osteoblasts, and the presence of YB does not change this behavior.

This information suggests that the ability of YB to promote bone healing cannot be simply and exclusively related to its activity on MSCs differentiation; this effect is probably related to a set of properties showed by YB, such as the anti-inflammatory and angiogenic properties. In conclusion, YB can be considered as an effective aid in bone regeneration and in bone healing, but it needs to be associated to other more specific and more targeted therapies, to combine and improve its therapeutic effects.

Author Contributions: G.B., M.T. and B.Z. designed and improved the research; G.B. and C.G. performed research; P.G. provided samples of Yunnan Baiyao; G.B., C.G. and L.F. analyzed data; G.B. and M.T. wrote the paper, B.Z. supervised the study.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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