Effects of Tricalcium Silicate Cements on Osteogenic Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells In Vitro

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ABSTRACT

Tricalcium silicate cements have been successfully employed in the biomedical field as bioactive bone and dentin substitutes, with widely acclaimed osteoactive properties. This research analyzed the effects of different tricalcium silicate cement formulations on the temporal osteoactivity profile of human bone marrow-derived mesenchymal stem cells (hMW-MSCs). These cells were exposed to 4 commercially-available tricalcium silicate cement formulations in osteogenic differentiation medium. After 1, 3, 7 and 10 days, quantitative real time-polymerase chain reaction and Western blotting were performed to detect the expression of target osteogenic markers ALP, RUNX2, OSX, OPN, MSX2, and OCN. After 3, 7, 14 and 21 days, alkaline phosphatase assay was performed to detect changes in intracellular enzyme level. Alizarin Red S assay was performed after 28 days to detect extracellular matrix mineralization. In the presence of tricalcium silicate cements, target osteogenic markers were downregulated at the mRNA and protein levels at all time-points. Intracellular alkaline phosphatase enzyme levels and extracellular mineralization of the experimental groups were not significantly different from the untreated control. Quantitative polymerase chain reaction results showed increases in downregulation of *RUNX2, OSX, MSX2* and *OCN* with increase in time of exposure to the tricalcium silicate cements, while *ALP* showed peak downregulation at day 7. For Western blotting, OSX, OPN, MSX2 and OCN showed increased downregulation with increased exposure time to the tested cements. Alkaline phosphatase enzyme levels generally declined after day 7. Based on these results, it is concluded that tricalcium silicate cements do not induce osteogenic differentiation of hBM-MSCs *in vitro*.

Keywords: bone marrow; human origin; mesenchymal stem cells; osteogenic differentiation; tricalcium silicate cements

1.   Introduction

Bioactivity is one of the most coveted properties of tricalcium silicate cements (TSCs) that is responsible for the continuously-expanding clinical applications of these cements in biomedical tissue engineering. By definition, bioactivity is the ability of a biomaterial to induce a specific biologic response [1]. Tricalcium silicate cements are hydraulic cements wherein their major components, calcium di- and tri-silicates, react with water to form calcium silicate hydrate and calcium hydroxide [2]. The water that is required for these reactions may be derived *in vivo* from interstitial tissue fluid in the vicinity of the area of application of the TSCs.

Of particular interest to clinicians and researchers is the ability of TSCs to induce osteogenic responses when they are applied to bone defects (i.e. osteoactivity). The literature is replete with studies addressing the osteoactivity of TSCs with the overall results being supportive of their osteogenic potential [3-11]. However, a closer inspection of this body of literature raises important concerns. For instance, the aforementioned studies employed cell lines from different species to study the osteogenic properties of TSCs. While consensus on the cytotoxicity of a biomaterial may be reached because cytotoxicity ranking is preserved among different cell lines [12-14], the same cannot be extended to cell differentiation studies, since different cell lines react differently to the same stimulus [4, 12]. Intraspecies variation is another salient issue; for example, murine MSCs not only from differ from human MSCs, but also differ among various strains in their genetic marker expressions and culture behavior [15-17]. Another source of confusion arises from the frequent use of fully- or partially-differentiated cell lines with established secretory functions and hard tissue forming potential for analysis of the osteogenic differentiation potential of a biomaterial. Results from these studies only show how well-differentiated cells perform their normal physiologic secretory functions in presence of the

biomaterial of interest; they do not provide information on how that biomaterial affects the differentiation of those cells from their immature counterparts.

Bone marrow is a promising source of mesenchymal stem cells (MSCs) for a broad range of cellular therapies in regenerative medicine. Human bone marrow-derived MSCs (hBM-MSCs) provide an excellent model for studying the *in vitro* osteoactivity of TSCs. They are among the most thoroughly characterized cell lines in the field of regenerative medicine [18-21]. These cells are pluripotent, undifferentiated adult stem cells with multi-lineage differentiation potential [22-24]. They may be harvested from the bone marrow relatively easily [25], and have the ability to differentiate into mature, specialized cells with chondrogenic, osteogenic and adipogenic lineages [22-24]. To the best of our knowledge, the osteoactive effects of TSCs on hBM-MSCs have not been studied previously. To address this gap in knowledge and to better understand the bioactivity of TSCs, the objective of the present study was to evaluate the effects of several commercial TSC formulations on osteogenic differentiation of hBM-MSCs. Two null hypotheses were tested: i) TSC exposure has no osteogenic effects on hBM-MSCs, and ii) there are no differences among the different commercial TSC formulations in their prospective influences on osteogenic differentiation of hBM-MSCs.

2.   Materials and methods

2.1   Materials

The main constituents and primary phases of the tested TSCs are summarized in Table I. White (WMTA) and gray (GMTA) ProRoot MTA (Dentsply Tulsa Dental Specialties, Tulsa, OK) were mixed with distilled water. White (WMTAP) and gray (GMTAP) MTA Plus (Avalon Biomed Inc, Bradenton, FL) were mixed with the proprietary hydrogel supplied by the manufacturer. These TSCs were mixed in a liquid to powder ratio of 0.3 according to their

respective manufacturer's instructions. The mixed materials were placed in pre-sterilized Teflon molds (5-mm diameter and 3-mm thick), covered with pre-sterilized Mylar sheets, and allowed to set in a 100% humidity chamber for 24 hours.

To avoid the initial cytotoxic effects of TSCs as demonstrated in a previous study [26], a previously-published aging/elution protocol based on the cements' specific cytotoxic profiles was adopted to render the cement discs non-cytotoxic prior to initiation of the experiment [26, 27]. According to this protocol, cement discs were sterilized by UV light for 4 hours, aseptically placed in their respective sterile Transwell® inserts (3.0 micron porous filter, Corning Inc., MA), and immersed in complete culture medium (CCM) for 2 weeks at 37°C in 100% humidity. The CCM consisted of alpha minimum essential medium (αMEM) and 20% fetal bovine serum (Atlanta Biologicals, GA), supplemented with 2 mmol/L L-glutamine (Lonza, Wakersville, MD) and 100 U/mL penicillin G/streptomycin sulfate (Invitrogen Corp, Carlsbad, CA). The culture medium was changed every 3 days to eliminate cytotoxic eluents that may interfere with their bioactive properties.

2.2   Cell culture

Previously characterized hBM-MSCs with CD73+/CD105+/CD45− immunophenotype [28] were used in the present study. The cells were received from the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White, through a grant from NCRR of the National Institute of Health, Grant #P40RR017447. This cell line has a subpopulation of small, rapidly-proliferating cells with high multi-differentiation potential that have been identified as rapidly self-renewing cells, among larger mature MSCs with limited differentiation potential and slow proliferation rate [29]. The multi-lineage potential of this cell line has been confirmed in previous studies [18]. For the current experiment, cells

from passages 4 to 6 were plated in CCM at $1x10^4$ cells/cm^2. After 24 hours, or after the cells reached ≈70%-80% confluence, Transwells® containing the pre-treated cement discs were placed in their respective wells, and the medium was changed to osteogenic differentiation medium (ODM). The latter consisted of the original CCM, supplemented with 50 mg/mL ascorbic acid, 10 mmol/L β-glycerophosphate, and 100 nmol/L dexamethasone (Sigma-Aldrich, St Louis, MO). The ODM was changed every 3 days.

2.3   RNA isolation and quantitative real time-polymerase chain reaction

Quantitative real time-polymerase chain reaction (qRT-PCR) was used to detect the relative expression of target markers of osteogenic differentiation among the test groups. The procedures were performed by isolating total RNA from hBM-MSCs after 1, 3, 7 and 10 days of exposure to the test materials. Total RNA was isolated and purified using QIAshredder and RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's recommended protocol for RNA purification from animal cells. The purity and quantity of the resultant RNA were assessed from 2-µL samples by using a NanoDrop1000 spectrophotometer (ThermoScientific, Wilmington, DE). Equal amounts of total RNA (0.1 mg RNA/mL) were then reverse-transcribed into single-stranded complementary DNA (cDNA) by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) in a thermal cycler by using the recommended settings (25°C for 10 min, 37°C for 120 min, 85°C for 5 min). The resultant cDNA was stored at -20°C until commencement of the qRT-PCR procedures.

For qRT-PCR, alkaline phosphatase (*ALP*), osteocalcin (*OCN*), osteopontin (*OPN*), Runt-related transcription factor 2 (*RUNX2*), osterix (*OSX*) and muscle segment homeobox 2 (*MSX2*) were selected as target markers for osteogenic differentiation. Untreated hBM-MSCs in ODM (designated as MM) were assigned as the experimental control. The cDNA was mixed with

Taqman® PCR universal master mix II (Invitrogen Corp.), added in 20 µL portions to 96-well custom TaqMan array standard plates preloaded with the pre-designated primers and housekeeping gene, loaded into a 7300 Real Time PCR System (Applied Biosystems), and run in a regular cycle. Each cycle consisted of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of denaturing/annealing at 95°C for 15 sec followed by 60°C for 1 min. One hundred nanograms of cDNA were loaded in each 20 µL reaction. Each specimen was run in triplicates, with 18S rRNA used as the housekeeping gene. Relative quantification of gene expression was performed by using the comparative threshold cycle method ($\Delta\Delta C_T$), in which the expression level of each target marker was normalized to the 18S rRNA endogenous control used as an active reference. Data output was expressed as fold changes of mRNA expression levels, given by $2^{-\Delta\Delta C_T}$ [30]. Expression profiles of target gene markers were compared among test groups.

2.4   Western blotting

Western blotting was used to analyze the expression levels of OCN, OPN, OSX, MSX2 and RUNX2 proteins following exposure of the hBM-MSCs to the test TSCs. After 1, 3, 7 and 10 days, cells exposed to different treatments were lysed using Radio Immuno Precipitation Assay buffer with a protease inhibitor cocktail (Sigma-Aldrich). Bicinchoninic acid assay was used to determine the protein concentration per specimen using bovine serum albumin as a standard. Protein denaturing and reduction was accomplished by adding 4X Laemmli buffer (Bio-Rad Laboratories, Hercules, CA) to the specimens, after standardization of the total amount of protein to be loaded, followed by boiling the samples at 100°C for 5 min. Forty microliter specimens were loaded onto 18-well 4–20% gradient Criterion XT Bis-Tris Gel (Bio-Rad) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run for 1 hour at 150 volts on a Criterion™ electrophoresis cell (Bio-Rad) with β-actin (43 kDa) as the loading

control. The proteins were transferred from the gel to a PVDF membrane (Immobilon-FL PVDF, 0.45μm/ Immobilon-PSQ Membrane, PVDF, 0.2 μm, Millipore, MA) using a Criterion™ Blotter and PowerPac™ HC power supply (Bio-Rad) running at 100 volts for 30 min. The membranes were blocked with Odyssey® blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 hour, and the blocked membranes were incubated with the primary antibodies diluted in Odyssey® blocking buffer overnight. Table II represents the target primary antibodies, molecular weights in kDa and the dilutions used. The membranes were then washed at room temperature in phosphate-buffered saline (PBS) and 0.1% Tween 20 (PBST) with gentle shaking. Fluorescent-labeled secondary antibodies, IRDye® 800CW Goat anti-Mouse IgG2b-Specific and 680LT Goat anti-Rabbit IgG (H + L) infrared dyes (LI-COR), were diluted with Odyssey® blocking buffer in the dilutions listed in Table II. The membranes were incubated with the diluted secondary antibodies for 60 min in the dark, at room temperature with gentle shaking. The membranes were then washed with PBST for 4 times and after a final wash with PBS, they were scanned with an Odyssey® CLx infrared imager using the standard scanning parameters. Experiment was conducted in triplicates. Relative protein expression analyses were performed using the Image Studio Lite software (LI-COR).

2.5   Alkaline phosphatase enzyme activity

Intracellular release of ALP enzyme was assessed using the Quantichrom ALP assay kit (Bioassay Systems, Hayward, CA). Colorimetric determination was based on hydrolysis of p-nitrophenyl phosphate by ALP into inorganic phosphate and p-nitrophenol, a yellow-colored product [31]. After 3, 7, 14 and 21 days of exposure, the cement discs and their respective Transwell® inserts were retrieved. The cells were washed with PBS, lysed with 0.2% Triton X-100 for 20 min. Working solution was added to a clear-bottomed 96 well plate (150 μL/well),

and cell lysate was quickly added to the working solution using a multi-channel pipettor. Absorbance of p-nitrophenol at 405 nm was recorded every min for a maximum of 16 min by using a microplate reader. The level of ALP activity of the cell lysate in IU/L was calculated from the equation:

$$\frac{(ODSAMPLE\ t - ODSAMPLE\ o) \cdot Reaction\ Vol\ x\ 35.3}{(ODCALIBRATOR - ODH2O) \cdot Sample\ Vol \cdot t}$$

where *OD* is the optical density at 405 nm, and *t* is the time in min. *ODSample 0* is the optical density at 0 min. The resultant data were plotted on a graph, and the peak value for each material was selected and compared with the other materials.

2.6   Alizarin red S assay

Extracellular matrix mineralization was assessed both qualitatively and quantitatively by Alizarin Red S assay [32]. Alizarin red S (AR-S) stains calcium-rich deposits (nodules) produced by cultured cells; the dye may be extracted from the stained nodules and assayed by spectrophotometry. After the hBM-MSCs were exposed to cement discs in ODM for 28 days, the cement discs were retrieved and cells were washed with PBS, fixed in 10% formaldehyde and stained with AR-S (40 mmol/L solution, pH 4.2) for 20 min at room temperature under shaking. Images of the specimens were taken for qualitative assessment of the stained nodules. The stained nodules were then incubated in 10% acetic acid for 30 min and neutralized with 10% ammonium hydroxide. The supernatant was pipetted into a clear-bottom 96-well plate, and the absorbance of the supernatants was determined at OD = 405 nm. The level of AR-S staining in the specimens (mg/L) was determined according to a linear regression equation derived from a pre-equilibration standard curve of known AR-S concentrations.

2.7   Statistical analyses

For qRT-PCR, the effect of materials on expression of each osteogenic marker was analyzed separately for each time-point by using one-factor analysis of variance (ANOVA) and post-hoc Tukey test or their nonparametric equivalents. Similar tests were used for analyzing the protein levels obtained from Western blotting, as well as the results obtained from the ALP enzyme assay and the Alizarin red assay. Parametric versions of these tests were used after evaluation of the normality (Shapiro–Wilk test) and equal variance assumptions (modified Levene test) of the individual data tests. If those assumptions were violated, the data were nonlinearly-transformed to satisfy those assumptions before using parametric testing methods. If those assumptions remained violated after nonlinear transformation, the original data set was analyzed using Kruskal–Wallis ANOVA and Dunn's multiple comparison tests. Statistical significances for all analyses were set at $\alpha = 0.05$.

3.   Results

3.1   qRT-PCR

A detailed presentation of the fold regulation of the expression of target mineralization markers can be found in Figure 1. Expression of *ALP* was significantly downregulated at the 4 time-points in all treatment groups compared to the MM control, with maximum downregulation noted at day 7 for all treatment groups. No significant differences were found between *ALP* expression in treatment groups except between GMTA and WMTA at days 7 and 10, where *ALP* expression was significantly more downregulated in WMTA compared to GMTA and GMTAP. Expression of *RUNX2* was significantly downregulated for all treatment groups at all time-points compared to the MM control, except for day 3, where the downregulation was not statistically significant for all groups except for WMTA. White ProRoot MTA (WMTA) also showed significantly more downregulation of *RUNX2* expression at day 7 compared to other treatment

groups. At day 10, *RUNX2* expression was significantly more downregulated in WMTA when compared to GMTAP and WMTAP, but not to GMTA. Expression of *OSX* was significantly downregulated in WMTAP only at day 1, in GMTAP at days 1 and 3, in WMTA at days 1, 3 and 7, and in GMTA at all time-points. Expression of *OPN* in all treatment groups was significantly downregulated compared to the MM control at days 1, 3 and 7. At day 10, no differences were found between treatment groups and the MM control except for WMTAP, where *OPN* expression was significantly downregulated compared to the control. The other 3 groups were not significantly different from the MM control or WMTAP. Expression of *MSX2* was significantly downregulated in all treatment groups at day 1. At day 3, *MSX2* was downregulated in all treatment groups except for WMTAP, which was not different from both the MM control and the other treatment groups. At days 7 and 10, all groups showed significant downregulation of *MSX2* compared to the MM control. Expression of *OCN* was significantly downregulated in all treatment groups at all time-points compared to the MM control. No difference in *OCN* expression was identified among treatment groups at each time-point except between WMTAP and WMTA at day 3.

3.2   Western blotting

Figure 2 shows the results of the Western blotting. Expression of RUNX2 among groups was not significantly different at all time-points. Nevertheless, peak values were noted at day 7, after which the protein expression level declined at day 10. For OSX, no differences were found at day 1 and 7 among groups, while WMTAP was significantly downregulated at day 3. All groups were significantly downregulated at day 10. For OPN, only GMTA was significantly downregulated at day 1. No differences were noted among all groups at day 3. WMTA and GMTAP were significantly downregulated at day 7. All groups except GMTA were significantly

downregulated at day 10. For MSX2, no differences were found among groups at day 1, while significant downregulation was found in groups GMTA, GMTAP and WMTAP at day 3. MSX2 expression was not detected at days 7 and 10. For OCN, GMTAP was significantly downregulated at day 1, WMTAP at day 3, WMTA at day 7. No difference was found among all groups at day 10.

3.3   ALP assay

Figure 3 represent the expression of ALP by the MM control and TSC groups over the 4 time-periods. At day 3, ALP levels in all treatment groups were significantly less than both the CCM and MM controls. At day 7, ALP levels in all treatment groups were not significantly different from MM except for WMTAP. At day 14, ALP levels dropped compared with the activity observed at day 7. The ALP levels in all treatment groups were not significantly different from MM but significantly higher than CCM. Further reduction in ALP activity was observed for all experimental groups at day 21, with the results not significantly different from either control group.

3.4   Alizarin red S assay

After 28 days of incubation with the TSCs, WMTAP and GMTAP showed higher AR-S levels than WMTA, GMTA and the MM control, but the difference was not statistically significant (Figure 4A). All treatment groups showed significantly higher AR-S levels compared to the CCM control (Figures 4A and 4B).

4.   Discussion

Human BM-MSCs were employed for the present study due to their robust stemness, pleuripotency, relative ease of harvesting and their clinical significance [18, 20, 25]. Previous

studies have reported that TSCs increase the osteogenic potential of osteoblast precursors [33], pre-odontoblasts [34], human osteosarcoma cells [3], periodontal ligament and gingival fibroblast cells [4], osteoblasts [5], osteoblast precursor cells [9], dental pulp stem cells [10], human tooth germ stem cells [35]. To our knowledge, this is the first study that examined the effects of TSCs on the osteoactivity of a pleuripotent, human adult MSC cell line derived from the bone marrow.

The present study employed a multi-parameter approach to study the temporal expression profiles of osteogenic differentiation determinants by tracking the four major molecular events in osteogenesis: mRNA expression of osteogenic markers using qRT-PCR, protein expression using Western blotting, and intracellular ALP enzyme level, all at strategic time points following exposure to the test cements. This was followed by quantification of extracellular calcium deposition and matrix mineralization using AR-S assay after 28 days of exposure to the test cements. This enabled us to monitor the intra- and extra-cellular events that altered the translational, transcriptional, enzymatic and hard tissue forming levels over the length of the experiment.

The following 6 markers, ALP, RUNX2, OCN, OPN, MSX2 and OSX were chosen for examination of osteogenic differentiation in the present study. ALP appears to be intimately related to pre-osseous cellular metabolism and to the elaboration of a calcifiable bone matrix [36]. RUNX2 is a transcription factor involved in osteoblastic differentiation and skeletal morphogenesis. This transcription factor has been shown to effectuate the expression of OSX, collagen type 1 alpha-1 (Col1a1), OCN and OPN by binding to the promoters of these genes [37]. Generally, RUNX2, ALP, Col1a1, transforming growth factor-beta 1 (TGF-β1), osteonectin and bone morphogenetic protein-2 are known to be early markers of osteoblastic

differentiation, whereas OCN and OPN are expressed later in the differentiation process [38]. MSX2 promotes osteoblast differentiation and bone formation by upregulating osterix in a RUNX-dependent manner [39]. OSX is a zinc finger-containing transcription factor, specifically expressed in all developing bones. The protein produced is a bone specific transcription factor and is required for osteoblast differentiation and bone formation [40].

The presence study found no evidence of osteoactivity of TSCs on hBM-MSCs up to 28 days of exposure of these cells to the tested cements. On the contrary, results derived from mRNA and protein analyses indicate that exposure of hBM-MSCs to TSCs over the designated time periods inhibited osteogenic differentiation when compared with cells that were not exposed to TSCs. This was manifested by downregulation of all osteogenic markers tested both in the transcription and the translation phases. The results of ALP and AR-S assays also confirmed the absence of any osteogenic effect of TSCs on hBM-MSCs. Thus the first null hypothesis that TSC exposure has no osteogenic effects on hBM-MSCs cannot be rejected. The present study also found no notable differences in the osteogenic effects of different formulations of TSCs on hBM-MSCs. Thus, the second null hypothesis that there are no differences among the different commercial TSC formulations in their prospective influences on osteogenic differentiation of hBM-MSCs cannot be rejected.

Interpretation of the results of the present study requires in-depth understanding of the major molecular regulators of BM-MSC differentiation. Because of the significance of BM-MSCs in the field of regenerative medicine, these cells have been studied extensively. Two of those regulators are of particular interest to the present work: the Wnt/β-catenin signaling pathway and the TGF-β superfamily pathways and their roles in MSC differentiation.

The Wnt/β-catenin pathway has been associated in skeletogenesis, bone development and homeostasis [41]. Wnt signaling pathway has differential effects on osteogenesis, which are dependent on the stage of cellular differentiation of the recipient cells [42]. Activation of Wnt/β-catenin signaling pathway has been shown to prevent transdifferentiation of osteoblast to chondrocytes [43], while at the same time inhibiting osteogenic differentiation of BM-MSCs [44]. In a recent article investigating the relationship between the intracellular pH and the Wnt/β-catenin signaling in cancer cells, cross-talk was found between intracellular pH and Wnt signaling pathway activation [45].

A more recent article studied the effects of silicon (Si) on the sonic hedgehog and Wnt signaling pathways, as well as expression of osteogenic markers in BMSCs. *AXIN2 and β-catenin*, two WNT/SHH signaling related genes, were upregulated in response to Si [46]. Furthermore, Si counteracted the effect of cardamonin, a major Wnt signaling pathway inhibitor. A noteworthy observation in that study is that the Si concentration at which β-catenin was upregulated - the highest concentration (5 mM Si) corresponded to the least ALP activity and cell viability. These observations suggest that TSCs may activate the Wnt/β-catenin pathway partly due to the change of cellular microenvironmental pH and partly due to Si release, leading to inhibition of osteogenic differentiation of BM-MSCs. It should be noted that all the TSCs examined produced highly alkaline extracellular environments (pH $\geq$ 10) after setting by the release of $Ca(OH)_2$ from the set cements.

Another major regulator of MSC differentiation is the TGF-β superfamily signaling pathway. TGF-β signaling pathway promotes cartilage-specific gene expression through intracellular signaling cascades involving SMAD proteins, the mitogen activated protein (MAP) kinases, p38, extracellular-signal regulated kinase (ERK)-1, and c-Jun N-terminal kinase (JNK)

[47]. Tuli and colleagues [48] demonstrated that TGF-β_1 treatment initiates and maintains chondrogenesis of MSCs, through the differential chondrostimulatory activities of p38, ERK-1 and JNK in the early steps of MSC chondrogenesis. Transcriptional profiling of MSCs revealed that TGF-β was significant in chondrogenesis, but axonal guidance pathway was significant in adipogenesis and osteogenesis. Furthermore, inhibition of ALK-5 (TGF-β type I receptor)–mediated TGF-β signaling resulted in enhanced adipogenic and osteogenic differentiation, but a complete lack of chondrogenic differentiation [49]. Previous studies have shown that TSCs release TGF-β from gingival fibroblasts [50, 51]. Thus, inhibition of osteogenic differentiation of MSCs by activation of TGF- β signaling pathway after exposure to TSCs may be a potential consequence.

5.   Conclusion

In conclusion, the present study did not find any *in vitro* evidence that TSCs can induce osteogenic differentiation of hBM-MSCs. The results suggest that the previously-reported osteoactivity of TSCs may be dependent upon the cellular differentiation stage, with possible connection to the activation of Wnt canonical and TGF-β signaling pathways in MSCs. This also suggests that fully- or partially-differentiated cell types with established secretory functions such as osteoblasts and pre-osteoblasts may play a more substantial role in the reported *in vivo* osteogenicity of TSCs, rather than the osteogenic differentiation of pluripotent stem cells. Future research should be directed at examining the direct effects of TSCs on Wnt canonical and TGF-β signaling pathways. Identification of the roles of various subpopulations of bone/hard tissue progenitor cells in bone formation/healing is also of prime importance. Research in this direction is in order.

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Table I: Comparison of gray and white varieties of ProRoot MTA and MTA Plus

Characteristics	White ProRoot MTA	Gray ProRoot MTA	White MTA Plus	Gray MTA Plus
Liquid	Water		Water-based gel with water-soluble thickening agents and polymers [a]	
Powder:liquid ratio (by weight)	3:1		Variable from 1:1 to 4:1 depending on indication	
Primary Phases	$3CaO.SiO_2$ $2CaO.SiO_2$ Bi_2O_3 [b] $3CaO·Al_2O_3$ $CaSO_4$	$3CaO.SiO_2$ $2CaO.SiO_2$ Bi_2O_3 [b] $3CaO·Al_2O_3$ $CaSO_4$ $Ca_2(Al,Fe)_2O_5$ [c]	$3CaO.SiO_2$ $2CaO.SiO_2$ Bi_2O_3 [b] $3CaO·Al_2O_3$ $CaSO_4$	$3CaO.SiO_2$ $2CaO.SiO_2$ Bi_2O_3 [b] $3CaO·Al_2O_3$ $CaSO_4$ $Ca_2(Al,Fe)_2O_5$ [c]

[a] contents are GRAS (generally regarded as safe); [b] bismuth oxide added for radiopacity;
[c] calcium aluminoferrite is only present in the gray variety of both cements. It is involved in the hydraulic phase and is more radiopaque than calcium silicate hydrate.

Table II: List of antibodies used for Western blot analysis including molecular weights, isotypes, clonality, localization, secondary antibodies and concentrations used

Name	Code	Description	Clonality	Isotype	Concentration	Molecular weight	Cellular localization	Secondary antibody	Additional bands
Anti MSX2	ab69058	Rabbit polyclonal to Msx2/Hox8	polyclonal	IgG	1 μg/mL	29 kDa	nuclear	Goat polyclonal to rabbit IgG at 1/3000 dilution	48, 55
Anti-RUNX2	ab23981	Rabbit polyclonal to RUNX2	polyclonal	IgG	1 μg/mL	57 kDa	nuclear	Goat polyclonal to rabbit IgG at 1/10000 dilution	
Anti-Sp7 / osterix antibody	ab22552	Rabbit polyclonal to Sp7/osterix – ChIP grade	polyclonal	IgG	2.5 μg/mL	46 kDa	nuclear	Goat anti-rabbit IgG	
Anti-osteopontin antibody	ab91655	Rabbit monoclonal [EPR3688] to osteopontin	monoclonal	IgG	1/1000	35 kDa	secreted	HRP-conjugated goat anti-rabbit at 1/2000 dilution	
Anti-osteocalcin antibody	ab13420	Mouse monoclonal [OCG3] to osteocalcin	monoclonal	IgG3	3 μg/mL	12 kDa	secreted	HRP-conjugated goat anti-mouse IgG polyclonal at 1/5000 dilution	
Anti-actin antibody	ab3280	Mouse monoclonal [ACTN05 (C4)] to actin	monoclonal	IgG1	0.5 μg/mL	42 kDa	cytoplasm > cytoskeleton	HRP-conjugated goat anti-mouse IgG polyclonal at 1/5000 dilution	

Figure legends

Figure 1: Temporal profiles of mRNA expression of target osteogenic markers in hBM-MSCs after exposure to different TSCs in osteogenic differentiation medium, using qRT-PCR with 18S as the endogenous control. Groups connected with a horizontal bar are not significantly different (p>0.05). WMTAP: White MTA Plus; GMTAP: Gray MTA Plus; WMTA: White MTA; GMTA: Gray MTA; MM: untreated cells in osteogenic differentiation medium.

Figure 2: Temporal profiling of the expression of target osteogenic marker proteins in hBM-MSCs after exposure to different TSCs in osteogenic differentiation medium. Figures on the left side of each figure-pair for the respective protein marker are representative Western blot scans of each protein, with peak protein densities normalized to the experimental control at each time-point. Different letters in each row indicate statistical significance. Figures on the right side of each figure-pair for the respective protein marker are graphical representations of mean protein densities in arbitrary units at each time-point. Groups connected with a horizontal bar are not significantly different (p>0.05). WMTAP: White MTA Plus; GMTAP: Gray MTA Plus; WMTA: White MTA; GMTA: Gray MTA; MM: untreated cells in osteogenic differentiation medium.

Figure 3: Intracellular alkaline phosphatase (ALP) enzyme level in hBM-MSCs after exposure to different TSCs in osteogenic differentiation medium for 3, 7, 14 and 21 days. Groups connected with a horizontal bar are not significantly different (p>0.05). WMTAP: White MTA Plus; GMTAP: Gray MTA Plus; WMTA: White MTA; GMTA: Gray MTA; MM: untreated cells in osteogenic differentiation medium; CCM: untreated cells in complete culture medium.
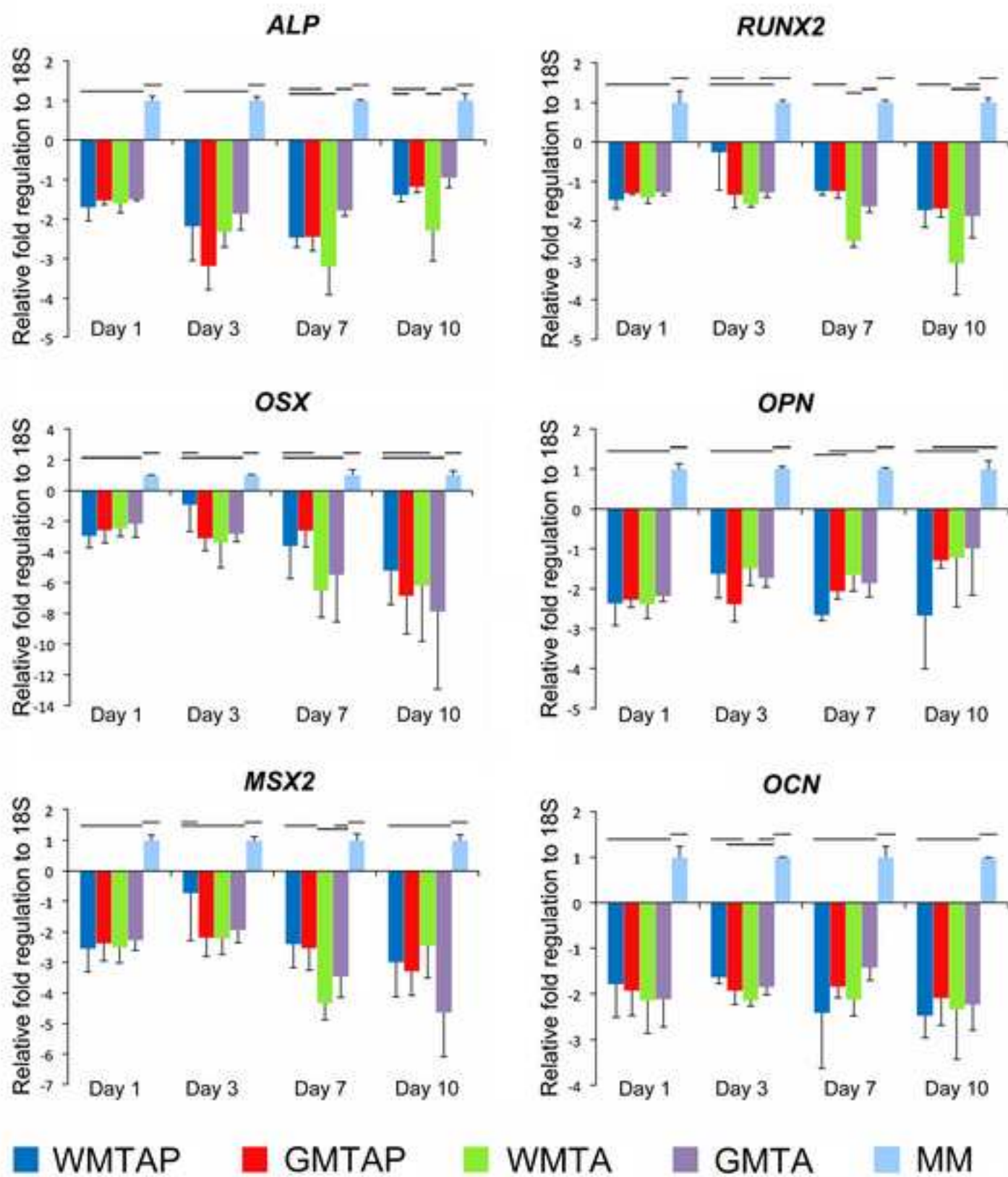
Figure 4: **A**. Alizarin red-S stain level in the extracellular matrix secreted by hBM-MSCs after 28 days of exposure to different TSCs in osteogenic differentiation medium. Groups connected

with a horizontal bar are not significantly different (p>0.05). **B**. Representative images showing macroscopic evaluation of Alizarin red-S staining in the specimens before stain extraction and quantification. WMTAP: White MTA Plus; GMTAP: Gray MTA Plus; WMTA: White MTA; GMTA: Gray MTA; MM: untreated cells in osteogenic differentiation medium; CCM: untreated cells in complete culture medium.
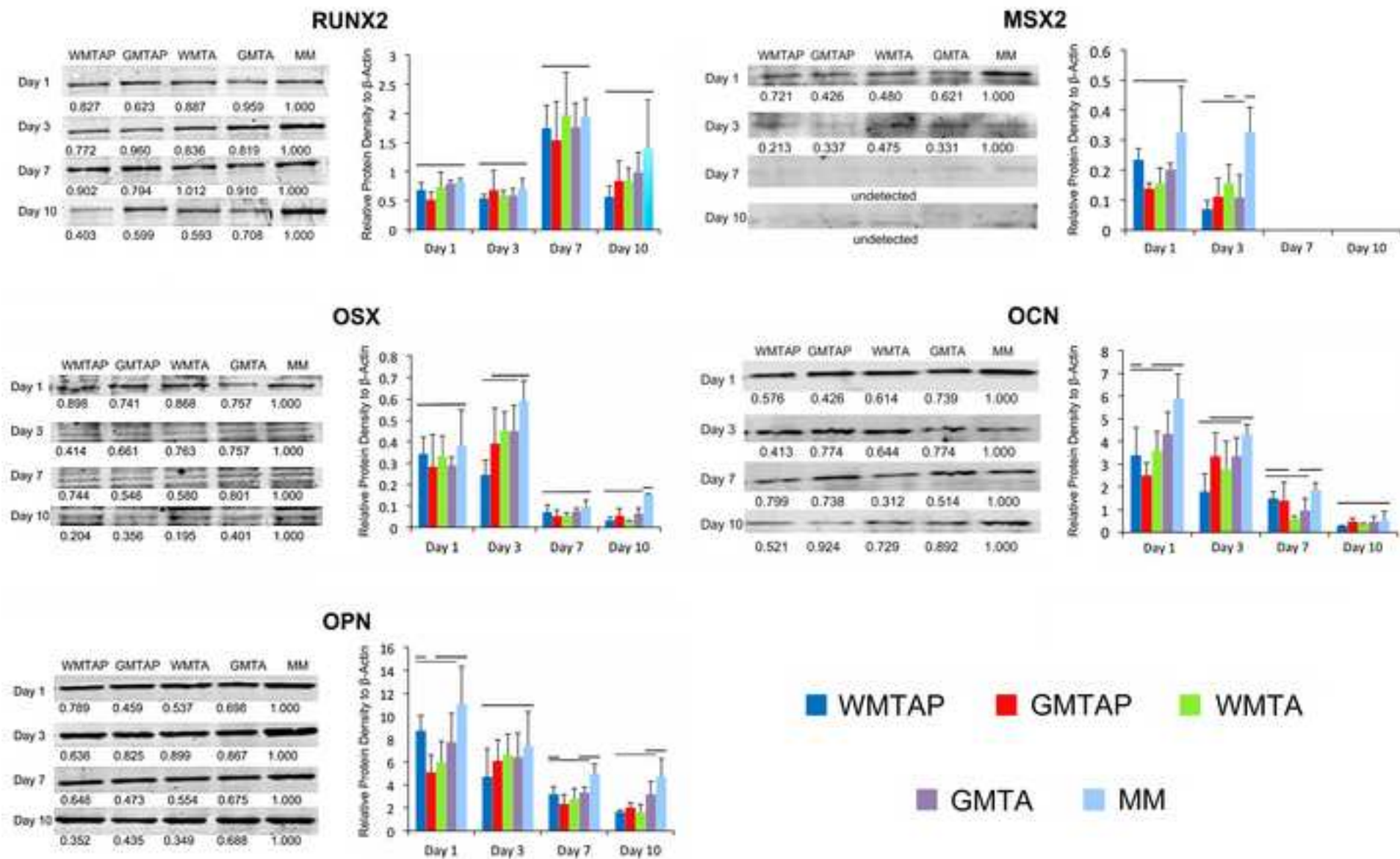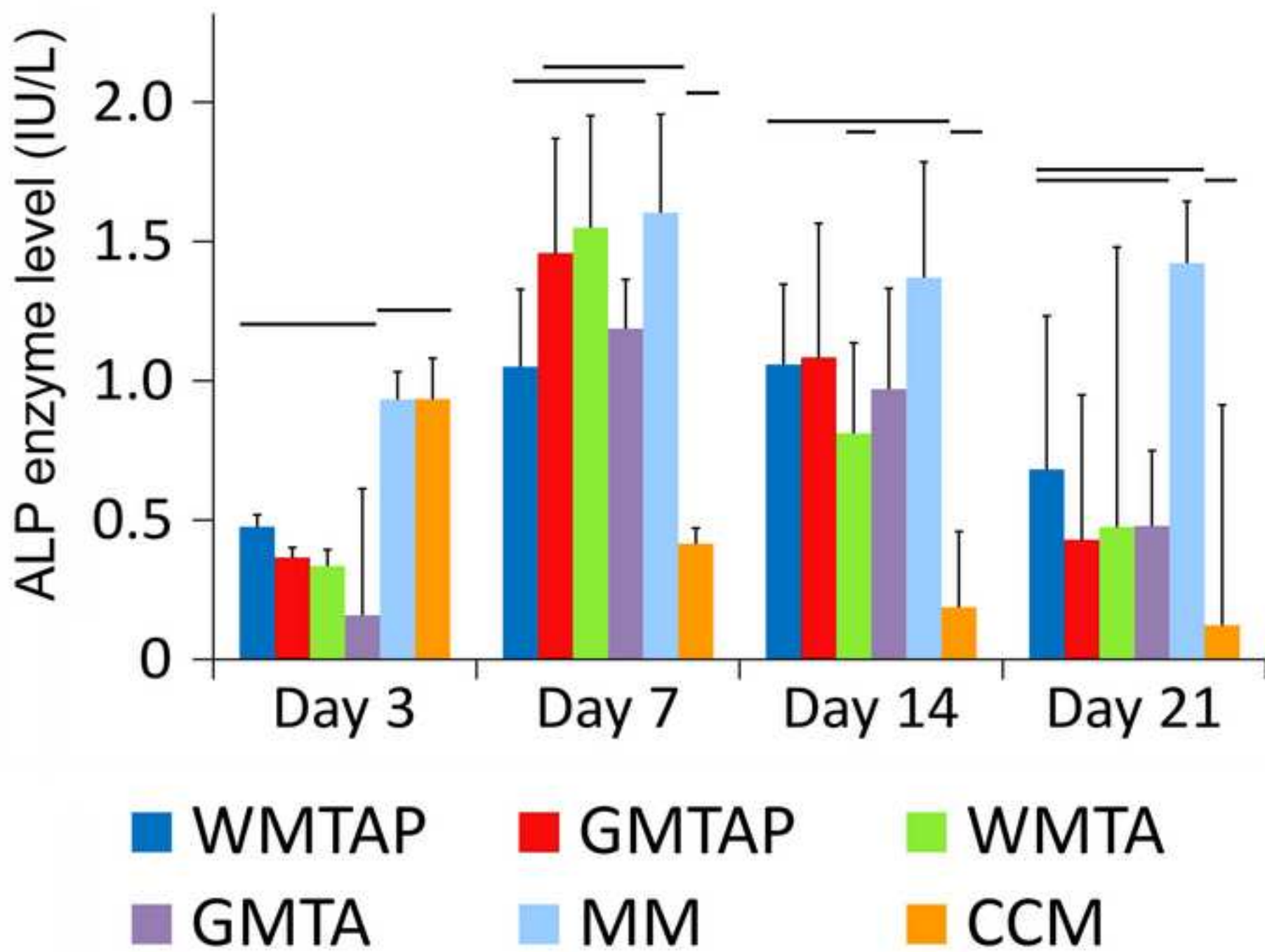
Figure 1

Figure 2

Figure 3

Figure 4