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Transient Receptor Potential Melastatin 3 and Intracellular Calcium in Natural Killer Cells in Multiple Sclerosis

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Abstract

Background: Natural killer (NK) cell phenotypes have reported to be implicated in the pathomechanism of Multiple Sclerosis (MS). Several investigators have observed reduced peripheral numbers, reduced cytotoxic activity, and altered $CD56^{Dim}$ and $CD56^{Bright}$ NK cell phenotypes. This current project, for the first time, investigates the NK cell cytotoxicity, calcium mobilisation and transient receptor potential melastatin 3 (TRPM3) surface expression. **Methods:** NK cell cytotoxic activity and calcium signaling were examined in $CD56^{Dim}$ and $CD56^{Bright}$ NK cells before and after stimulation using Ionomycin, Pregnenolone sulphate, 2-Aminoethoxydiphenyl borate and Thapsigargin. Purified NK cells were labelled with antibodies to determine TRPM3, CD69 and CD107a surface expression using flow cytometry. **Results:** Twenty-two MS patients and 22 healthy controls were recruited for this project. Twelve of the 22 previously received Alemtuzumab (Lemtrada®) and the remaining ten reported nil medication. We report TRPM3 was significantly increased in untreated MS patients compared with healthy controls and treated MS patients (p-value 0.034). There was a significant decrease in CD69 surface expression on $CD56^{Dim}$ NK cell phenotype for untreated MS patients (p-value 0.031) and treated MS patients (p-value 0.036). We report altered calcium mobilisation in $CD56^{Bright}$ NK cells and to a lesser extent $CD56^{Dim}$ NK cells between healthy controls, treated and untreated MS patients. **Conclusion:** This investigation suggests variations in TRPM3 expression and calcium mobilisation of NK cells may be implicated in the pathogenesis of MS. Further investigation is required to determine the mechanism by which alemtuzumab

alters calcium signaling in NK cells.

Keywords

Natural Killer Cells, Multiple Sclerosis, Calcium Signalling, Transient Receptor Potential Melastatin 3 Ion Channels

1. Introduction

Multiple Sclerosis (MS) is an inflammatory disease resulting in demyelination within the central nervous system (CNS). The aetiology of MS is multifactorial with studies implicating genetic, environmental, and epigenetic factors that ultimately appear to cause immune dysregulation in both the innate and adaptive immune systems [1] [2]. MS is the most common cause of neurological disability in young adults aged 20 - 45 years of age [3] and is predominately diagnosed in women [4]. Currently, there is no single, curative treatment for MS and therapies aim to suppress immune response in the CNS [3].

Natural killer (NK) cells have recently attracted significant attention for being implicated in the pathogenesis of MS [1] [5] [6] [7]. NK cells are a subset of lymphocytes in the innate immune system that are responsible for eliminating invading pathogens and tumour cells, as well as regulating T cell immunity [8]. There are two main subsets of NK cells defined by surface expression of CD56 and CD16 surface markers. $CD56^{Bright}CD16^{Dim}$ NK cells have a high density surface expression and CD56 and are prominent in the production of cytokines. $CD56^{Dim}CD16^{Bright}$ NK cells produce higher levels of lytic proteins; therefore they have the ability to mediate cytotoxicity on target cells [9]. Previous investigations have reported significant reductions in the number of circulating NK cells, reduction in NK cell cytotoxic activity, and a reduction in the production of pro-inflammatory cytokines in MS [5] [6] [10] [11] [12] [13]. A previous study has shown that changes to $CD56^{Bright}$ NK cells enable autoreactive T cell survival that may contribute to lesion formation [14]. Furthermore, in one study NK cell functional activity was shown to diminish at the onset of clinical relapse, and normalised post relapse recovery [15].

Calcium (Ca^{2+}) mobilization and its regulation by ion channels are important in immune cell function [16]. In NK cells, polarisation of cytolytic granules and the release of lytic proteins are dependent on intracellular Ca^{2+} concentration [17], however the intracellular signalling mechanisms by which this is initiated and regulated requires further investigations.

The transient receptor potential (TRP) channel family comprise non-selective cation channels including Ca^{2+} permeable channels that play a role in the influx and transportation of intracellular Ca^{2+} [18]. There are six TRP subfamilies; TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPML (mucolipin, and TRPP (polycystin) [19] [20]. TRP ion channels are widely expressed on tissues and cells that become activated in response to various

stimuli in the cellular environment including pain, temperature, taste, pressure and vision [19]. TRPs have a role in store operated Ca^{2+} signalling in non-excitable cells including but not subjected to cells of the CNS [21]. TRP ion channels are expressed by multiple cell types throughout the body [18] including cells of the peripheral nervous system (PNS) and CNS [18] [22], and innate and adaptive immune system [16]. TRP ion channels have been implicated in neurological disease; therefore they pose as a potential therapeutic target [22]. TRP channels may have a role in the pathogenesis of MS [23] [24].

TRPM3 ion channels are transmembrane channels belonging to the melastatin subfamily [20]. There are seven isoforms identified in the human TRPM3 gene, including TRPM3α2 which has been characterised as having a Ca^{2+} conducting pore and play a role in store-operated Ca^{2+} signaling [25] [26]. TRPM3 has been proposed to play roles in a variety of physiological and pathophysiological processes including but not subjected to noxious heat sensation [27]. TRPM3 is expressed in several tissues including the kidneys, eyes, sensory neurons of the dorsal root ganglia, pancreatic β cells, and white matter of the CNS [26] [28]. More recently, it has been demonstrated that TRPM3 ion channels are expressed on the surface of both NK cells and B lymphocytes [29]. Previous research has documented TRPM2, TRPM4 and TRPM5 channel expression on lymphocyte subsets [30] [31]. However, there is limited literature on TRPM3 expression on NK cells as only two previous investigations using flow cytometry have reported TRPM3 [29] [32].

Whilst research to date suggests that NK cells may play a regulatory role in the pathogenesis of MS, the role of Ca^{2+} mobilisation in mediating NK cell cytotoxicity through Ca^{2+} specific receptors, such as TRPM3, has not been investigated. Moreover, the possible role of receptor/channel/Ca^{2+} interaction in NK cells from MS patients has not been investigated. Therefore the aim of this study was to conduct a case-controlled study to investigate NK cell cytotoxic activity and intracellular Ca^{2+} mobilisation in healthy controls (HC) compared to untreated MS patients and patients administered with alemtuzumab. In addition, we examined TRPM3 surface expression as a possible mechanism for altered NK cell function in MS patients.

Additionally, Lemtrada® (alemtuzumab) is a humanised monoclonal antibody against cell surface protein CD52 [33]. CD52 is expressed primarily on T and B lymphocytes, but also minimally expressed on NK cells [34]. The exact function of CD52 is speculation, however, it is a targeted protein for antibody-dependent cytotoxicity induced by alemtuzumab therapy [33]. Alemtuzumab is a novel treatment for NK cell neoplasms [34] and proposed as a treatment for MS since the 1990s [35] [36]. A previous investigation suggested NK cells are a key component to alemtuzumab-dependent cytokine release and lymphocyte depletion by antibody-dependent cell mediated cytotoxicity [37]. As alemtuzumab is believed to activate NK cell cytotoxicity, for this reason, we also aimed to examine the effect of alemtuzumab therapy on NK cell cytotoxicity, TRPM3 expression and Ca^{2+} mobilisation.

2. Methods

2.1. Participants

MS participants were recruited from specialist neurology outpatient clinics at a tertiary referral hospital, clinical trial outpatient clinics, and a previously established MS database three months prior to study commencement. This study was approved by the Griffith University Human Research Ethic Committee (MSC/18/13). MS patients were defined in accordance with the 2010 Revised McDonald diagnostic criteria [38]. Participants were recruited across all disease courses (clinically isolated syndrome, relapsing remitting, primary progressive, secondary progressive). Untreated MS participants were defined as those not currently on therapy for the treatment of MS, and were excluded if they had been on therapy for treatment of MS within the last six months. Participants in the treatment group were those previously treated with alemtuzumab (12 mg/dose), with inclusions for participants treated with both one course (5 days) or two courses (5 days + 3 days, 12 months following initial therapy). Participants in both MS groups were further excluded if they were taking other immunomodulatory medications. HC were recruited between the ages of 18 - 65 and upon clinical assessment were reported to be in good health. Participants were excluded if pregnant, breastfeeding, or had a history of smoking, another autoimmune disorder, psychosis, major depression, cardiovascular disease, thyroid disease or diabetes. Participants were excluded if they reported administration of medications that directly or indirectly effect TRPM3 activation and Ca^{2+} mobilisation.

Participants donated 80 mL of blood between 7:00 am and 11:00 am. Blood was collected into lithium-heparinised and ethylenediaminetetraacetic acid (EDTA) collection tubes. Peripheral blood mononuclear cells (PBMCs) and NK cells were isolated from 70 mL of whole blood while 10 mL was used for full blood analysis within six hours of blood collection. Pathology testing was performed to exclude concurrent inflammatory and chronic diseases and included full blood count, electrolytes, erythrocyte sedimentation rate (ESR), C-reactive protein, dehydroepiandrosterone, adrenocorticotropic hormone and cortisol. Clinical data collected included demographic details, age at onset, disease duration, number of relapses, disease course, prior medical history and exposures, expanded disability status scale (EDSS) and previous MS treatment.

2.2. Peripheral Blood Mononuclear Cell and Natural Killer Cell Isolation

PBMCs were isolated from whole blood via centrifugation over Ficoll density gradient medium (Ficoll GE Healthcare Bio-Sciences AB, Uppsala, Sweden). This was followed by immunomagnetic isolation of NK cells using EasySep NK cell enrichment kit (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Isolated NK cells were considered 89.5% ± 3.64 for HC and 90.4% ± 4.13 for MS.

2.3. Drug Stimulation of Natural Killer Cells

Isolated NK cells (1×10^6 cells/ml) were stimulated by incubation for four hours at 37˚C with 5% CO_2 in RPMI1640 supplemented with 10% fetal bovine serum (FBS) in the presence of the following stimulants at a final concentration of Ionomycin (1 μM), Pregnenolone Sulphate (PregS) (1.35 uM), PregS (1.35 μM) + 2-Aminoethoxydiphenyl borate (2-APB) (50 μM), and PregS (1.35 μM) + Thapsigargin (TG) (1.42 μM,) 2-ABP (50 μM), and μMTG (1.42 μM) (Bio-Techne, Tocris Bioscience, Sussex, UK, except Ionomycin purchased from Sigma, Mansfield, Australia). PregS reversibly activates TRPM3 leading to rapid influx in Ca^{2+} [39] while 2-APB acts to inhibit both IP3 receptors and TRP channels Ionomycin allows extracellular Ca^{2+} ions into the cytosol [32] while TG increases cytosolic Ca^{2+} through inhibition of the endoplasmic reticulum Ca^{2+} pump [40].

2.4. TRPM3 Immunophenotyping in Natural Killer Cells

TRPM3 expression on resting NK cells was identified as previously described [29] [32]. Isolated NK cells were incubated with primary fluorochrome labelled antibodies CD19-BV421, CD3-PerCP, CD56-BV421, CD16-APC Cy7 (purchased from Beckon Dickinson Bioscience, Miami, Florida, US) and TRPM3 primary antibody (purchased from Santa Cruz Biotechnology, Dallas, Texas, US) for 30 minutes at room temperature in the dark. Cells were washed and then incubated with TRPM3 secondary antibody (purchased from Santa Cruz Biotechnology, Dallas, Texas, US) for 30 minutes. NK cells were stimulated using the above protocol for four hours at 37˚C with 5% CO_2 in RPMI-1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Carlsbad, CA, USA).

Cells were stained with CD69, CD107a and TRPM3 primary antibody for 30 minutes to determine CD69, CD107a and TRPM3 receptors expression on $CD56^{Dim}$ and $CD56^{Bright}$ NK cell phenotypes. TruCount counting beads (BD Bioscience, San Jose, CA, USA) were used to calculate NK cell concentration as well as absolute cell counts and was determined using the manufacturer's instructions outline in the below formula. Cells were washed and resuspended in 200 μl of staining buffer (BD Bioscience, San Jose, CA, USA) and acquired using LSRFortessa X-20 (BD Bioscience, San Jose, CA, USA). Isotype controls were used to determine appropriate gating as previously described [32]. 20,000 events were recorded for each sample.

$$\frac{\text{number of events in cell region}}{\text{number of events in bead region}} \times \frac{\text{number of beads/test}}{\text{test volume}} \times \text{dilution factor} = \text{cell population concentration}$$

2.5. Cytoplasmic Calcium in Natural Killer Cells

Ca^{2+} flux was performed as previously described [29] [32]. Ca^{2+} signalling was measured in the presence of the following stimulants at a final concentration of 1 μM Ionomycin, 1.35 μM PregS, 1.35 μM PregS + 50 μM 2-APB, 1.35 μM PregS

+ 1.42 μM TG, 50 μM 2-ABP alone, and 1.42 μM TG alone. Assessment of Ca^{2+} influx dependent on TRPM3 was determined using PregS. CD56 and CD16 antibodies were used to determine NK cell phenotypes during flow cytometry. Cells were stimulated for five minutes and recorded in real time on flow cytometry.

2.6. Natural Killer Cell Cytotoxicity

Assessment of NK cell cytotoxic activity was performed as previously described [41]. Following NK cell isolation and labeling with 0.4% Paul Karl Horan (PKH-26) (Sigma-Aldrich, St Louis, MO, USA), NK cells were stimulated in the presence of the following drugs at a final concentration of 1 μM Ionomycin, 1.35 μM PregS, 1.35 μM PregS + 50 μM 2-APB, 1.35 μM PregS + 1.42 μM TG. Under stimulated conditions at effector to target ratio (E: T) of 1:1 was utilised. A baseline measurement of NK cell cytotoxic activity was also performed using E: T of 25:1, 12:1, 6:1 and 1:1. Cells were washed and plated with K562 cells. Cells were incubated for four hours at 37˚C with 5% CO_2 in RPMI-1640 supplemented with 10% FBS. Post incubation, cells were stained with Annexin V (2.5 μl/test) and 7-amino-acetinomycin (7-AAD) (2.5 μl/test) for determination of K562 viability [42]. K562 cell viability was then determined into 4 stages; live/viable K562 cells (AnnexinV−/7AAD−), K562 cells undergoing early stage apoptosis (AnnexinV+/7AAD−), late stage apoptosis (AnnexinV+/7AAD+), and dead K562 cells (AnnexinV−/7AAD+). K562 cell death was then calculated as previously described [42]. Flow cytometry was used to determine target cell apoptosis recording 20,000 events for each sample.

2.7. Data and Statistical Analysis

Data was exported from FacsDiva v8.1 and analysed using Flowjo software v10. Data was quantified, and statistical analysis was performed using SPSS 22.2 software. Shapiro-Wilk testing was performed for normality and Mann-Whitney U test was used to determine the relationship between healthy control and the MS group under each stimulant/condition with respect to TRPM3 expression, CD69 and CD107a surface expression on NK cell subsets, calcium signalling, area under the curve (AUC), and NK cell cytotoxic activity. Kruskal-Wallis test was then used to determine the relationship between healthy controls, the untreated MS group and MS group treated with alemtuzumab for the aforementioned measures. Statistical significance was set at <0.05. Demographic and patient data outputs are reported as mean ± SEM unless otherwise stated. ANOVA testing was performed on sociodemographic data and pathology results.

3. Results

3.1. Participants

A total of 44 participants were included; 22 HC, 12 untreated MS, and 10 MS patients treated with alemtuzumab. The sociodemographic and pathology data for

participants is summarised in Table 1 and Table 2 (Data not shown is summarised in Supplementary Table 1). There were no significant differences in age or

Table 1. Demographic data.

	HC	MS-Treated	MS-Untreated
N	22	22	12
Age (years)	39.4 ± 12.5	41.1 ± 15.2	46.5 ± 17.0
Female n (%)	6 (73)	17 (77)	8 (67)
Male n (%)	16 (27)	5 (23)	4 (33)

Data shown indicates mean ± standard deviation (SD). Gender results represented as number and percentage. Abbreviations: HC, healthy control; MS, multiple sclerosis.

Table 2. Pathology results.

	Healthy control		MS-Untreated			MS-Treated			MS Untreated vs Treated
	Mean	SD	Mean	SD	*p*-value vs HC	Mean	SD	*p*-value vs HC	*p*-value
Cholesterol mmolL	4.8	0.8	4.8	1.0	1	4.9	0.7	1	1
Haemoglobin gL	137.8	11.9	130.8	34.3	1	134.4	8.1	1	1
WBC × 10^9/L	5.9	1.3	6.7	1.4	0.27	4.8	1.2	0.079	0.003
Platelet × 10^9/L	263.7	71.2	278.3	37.2	1	243.0	43.4	1	0.49
Haematocrit	0.41	0.03	0.42	0.04	1	0.41	0.02	1	0.889
RBC × 10^{12}/L	4.65	0.39	4.68	0.53	1	4.59	0.35	1	1
MCV fL	88.8	4.4	90.0	2.0	1	88.1	4.0	1	0.744
Neutrophils × 10^9/L	3.50	0.89	4.29	1.36	0.150	3.42	1.12	1	0.208
Lymphocyte × 10^9/L	1.90	0.63	1.86	0.41	1	0.88	0.29	<0.001	<0.001
Monocyte × 10^9/L	0.33	0.10	0.39	0.13	0.348	0.29	0.09	0.992	0.095
Eosinophils × 10^9/L	0.15	0.08	0.16	0.09	1	0.16	0.10	1	1
Basophil × 10^9/L	0.03	0.02	0.04	0.02	0.916	0.02	0.01	0.577	0.140
ESR mmHr	13.5	10.2	19.6	14.6	0.409	11.6	8.4	1	0.314
DHEA Sulphate umolL	4.0	2.1	4.2	3.9	1	4.3	4.2	1	1
ACTH ngL	16.8	7.2	16.3	9.7	1	19.4	12.6	1	1
Cortisol nmolL	365.8	134.9	295.3	89.7	0.429	388.8	162.8	1	0.314
Hs CRP	2.04	2.21	3.33	4.92	0.733	1.57	1.12	1	0.550
sodium	136.3	2.2	136.7	3.3	1	137.4	2.4	0.772	0.803
Potassium	4.1	0.4	4.2	0.4	1	4.0	0.2	0.803	0.756
chloride	102.5	2.0	100.8	3.0	0.134	103.2	2.5	1	0.068
Bicarbonate	26.4	2.0	28.0	1.8	0.080	26.5	2.1	1	0.255
AnionGap	7.5	1.9	7.9	1.6	1	7.8	1.8	1	1

Data shown indicates mean, SD and *p*-values. Significant p-values highlighted in bold text. Abbreviations: MS: Multiple sclerosis, WBC: White blood cell, RBC: Red blood cell, MCV: Mean Corpuscular Volume, ESR: Erythrocyte sedimentation rate, CRP: C-reactive protein, DHEA: Dehydroepiandrosterone, ACTH: Adrenocorticotropic hormone.

gender between groups. There was no significant difference between the number of relapses, disease course, and EDSS between MS groups. The age of onset in the treated MS group was significantly lower than the age of onset in the untreated MS group (p-value 0.004). There was a significant difference in the white cell count (WCC) between untreated and treated MS patients (mean untreated 6.7×10^9/L vs mean treated 4.8×10^9/L, p-value 0.003). There was a significant reduction in lymphocyte count of the treated MS group compared to HC (mean HC 1.9×10^9/L vs mean treated 0.88×10^9/L, p-value <0.001), and a significant reduction in lymphocyte count in the treated MS group compared to untreated MS group (mean untreated 1.86×10^9/L vs mean treated 0.88×10^9/L, p-value <0.001). There was no significant difference in lymphocyte count between HC and the untreated MS group.

3.2. Natural Killer Cell Cytotoxicity

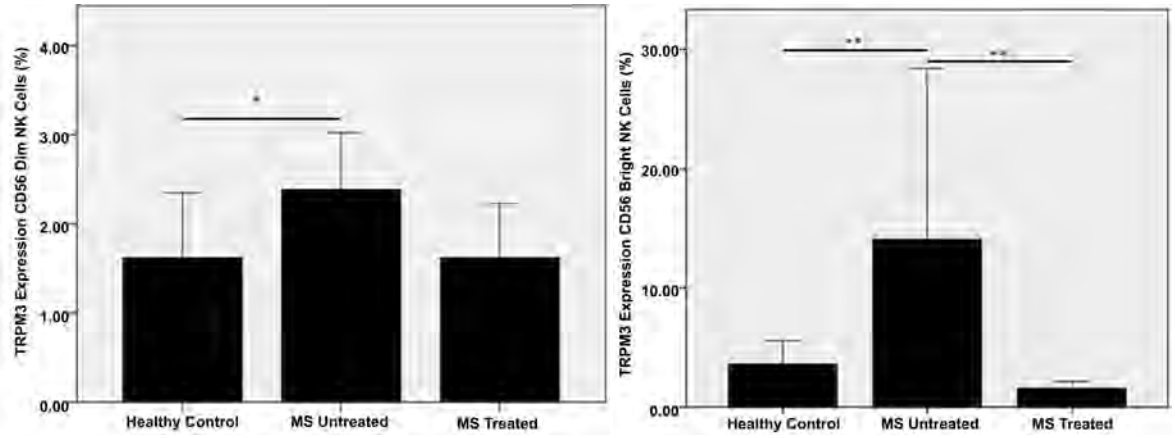
There was no significant difference in NK cell cytotoxic activity in HC compared with the MS group, nor was there any difference in NK cell cytotoxic activity between HC and MS subgroups, at any E:T (Data not shown). There was no significant difference seen with stimulation. A patient with Primary Progressive MS (PPMS) was removed from analysis as a possible outlier. With the removal of this individual, no significant difference in NK cell cytotoxicity remained.

3.3. TRPM3 Expression and Activation Markers on Natural Killer Cells

TRPM3 expression was significantly increased in $CD56^{Dim}$ NK cells in the untreated MS group compared with HC when under the stimulation of PregS (p-value 0.034) (**Figure 1**). Interestingly, in $CD56^{Dim}$ NK cells the treated MS group demonstrated no significant difference in TRPM3 expression than the HC group, suggesting TRPM3 may have reverted to normal expression with alemtuzumab treatment in these cells. A significant increase in TRPM3 expression was observed in $CD56^{Bright}$ NK cells of untreated MS patients when compared with HC and treated MS patients (**Figure 1(b)**).

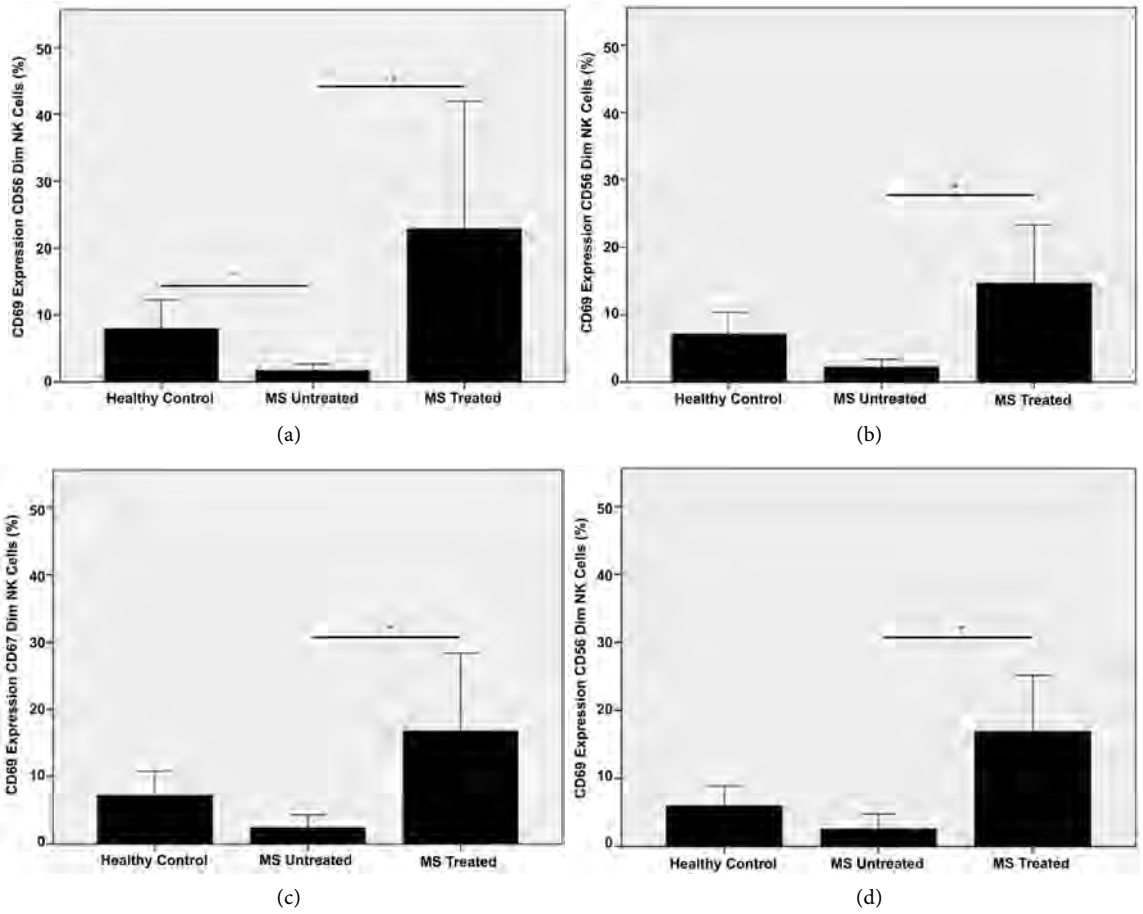
When unstimulated, CD69 expression was significantly lower on $CD56^{Dim}$ NK Cells of the untreated MS group when compared with both HC (p-value 0.031) and treated MS group (p-value 0.036) (**Figure 2(a)**).

There was no significant difference in CD69 expression in the $CD56^{Bright}$ cell population prior to drug stimulation (**Figure 3(a)**). Under stimulation with 2-ABP, there was a significant increase in $CD56^{Bright}$ NK cells expressing CD69 in the untreated MS group compared with HC (p-value 0.015) (**Figure 3(a)**). There was an increase in $CD56^{Bright}$ NK cells expressing CD69 in the untreated MS group compared with the treated MS group that approached, but did not achieve significance (p-value 0.081). Under stimulation with PregS alone, PregS + 2-ABP and 2-ABP alone, CD69 expression on $CD56^{Dim}$ NK cells was significantly higher in the treated MS group compared with the untreated MS group. Under these



(a) (b)

Figure 1. TRPM3 expression under Pregenolone Sulphate. (a) illustrates TRPM3 expression on $CD56^{Dim}$ NK cells; (b) illustrates TRPM3 expression on $CD56^{Bright}$ NK cells. Data are represented as mean ± SEM. Asterisks (*) represent statistical significance at p value < 0.05. Abbreviations: MS: Multiple sclerosis, NK: natural killer, TRPM3: Transient receptor potential melatastin.



(a) (b)

(c) (d)

Figure 2. Expression activation markers on $CD56^{Dim}$ NK cells. (a) $CD56^{Dim}$ NK Cells expressing CD69-US; (b) $CD56^{Dim}$ NK Cells expressing CD69-treated 2-ABP; (c) $CD56^{Dim}$ NK Cells expressing CD69-PregS; (d) $CD56^{Dim}$ NK Cells expressing CD69-PregS + 2-ABP. Data are represented as mean ± SEM. Asterisks (*) represent statistical significance at p value < 0.05. Abbreviations: PregS: Pregnenolone sulphate, TG: Thapsigargin, 2-ABP: 2-Aminoethoxydiphenyl borate, MS: Multiple sclerosis, NK: natural killer.

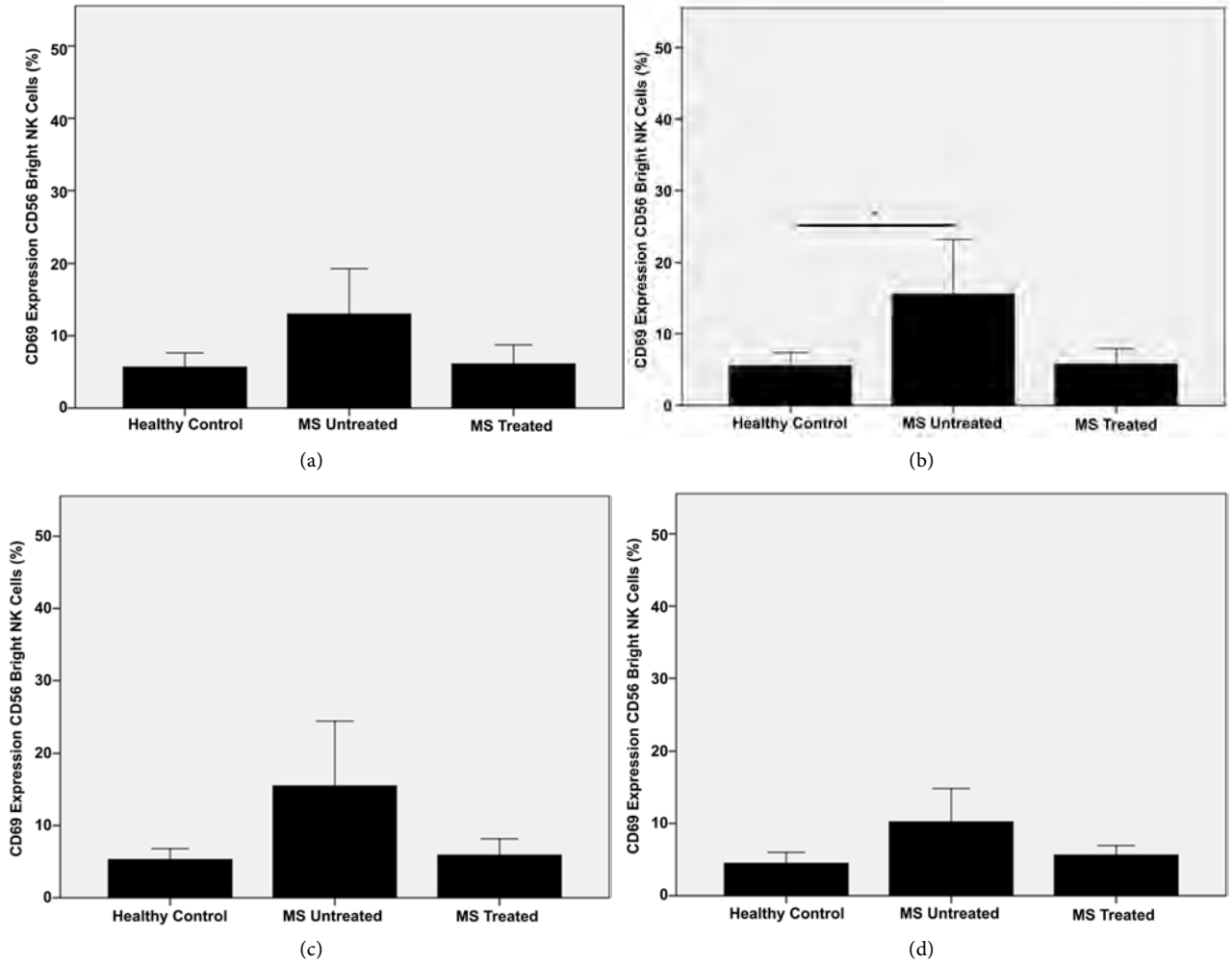


Figure 3. Expression activation markers on $CD56^{Bright}$ NK cells. (a) $CD56^{Bright}$ NK Cells expressing CD69-US; (b) $CD56^{Bright}$ NK Cells expressing CD69-treated 2-ABP; (c) $CD56^{Bright}$ NK Cells expressing CD69-PregS; (d) $CD56^{Bright}$ NK Cells expressing CD69-PregS + 2-ABP. Data are represented as mean ± SEM (*) represent statistical significance at p value < 0.05. Abbreviations: US: Unstimulated, PregS: Pregnenolone sulphate, 2-ABP: 2-Aminoethoxydiphenyl borate, MS: Multiple sclerosiss, NK: natural killer.

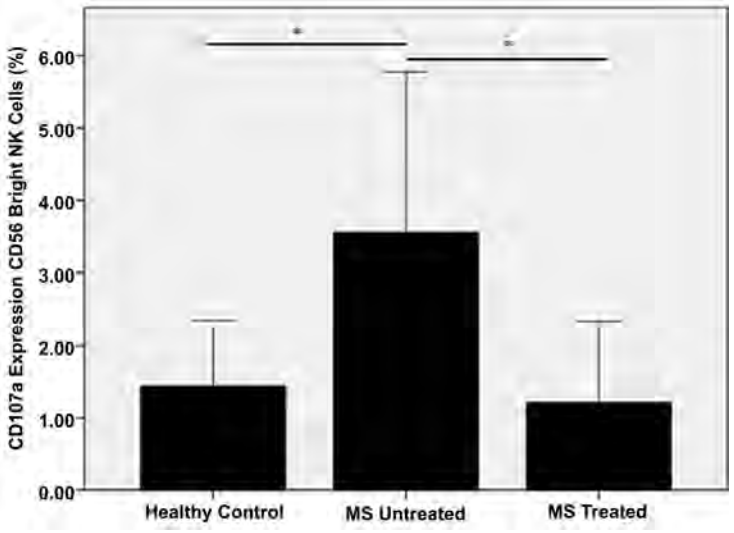
conditions, there was no significant difference between HC and the untreated MS group (Figure 2).

There was a significant increase in CD107a expression on $CD56^{Bright}$ NK cells in untreated MS patients compared with HC and treated MS patients (Figure 4(a)). There was no significant difference in CD107a expression on $CD56^{Dim}$ NK cells (Figure 4(b)).

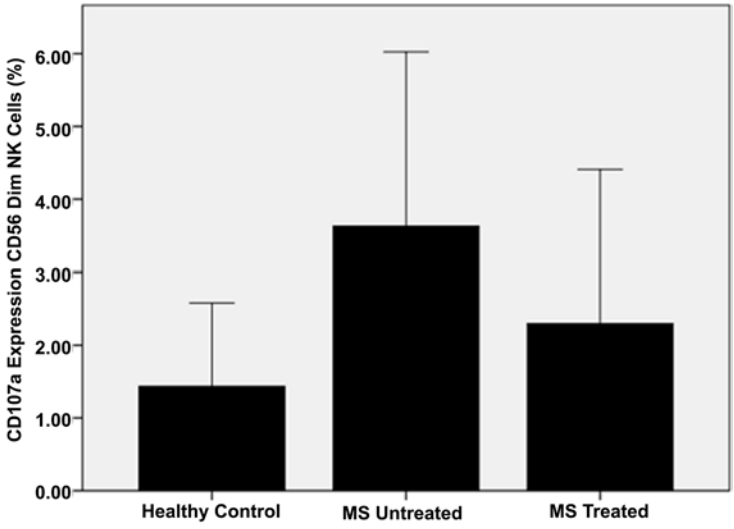
3.4. Intracellular Calcium Mobilisation

The There was no significant difference in Ca^{2+} signalling between subgroups with no stimulation. However, in the $CD56^{Bright}$ NK cell population there was a trend towards increased AUC in the treated MS group compared with HC (p value 0.052) (Figure 5(g)).

The $CD56^{Bright}$ NK cell population showed significantly increased AUC in the treated MS group compared with HC after 2-ABP (Figure 5(a)) and PregS +



(a)

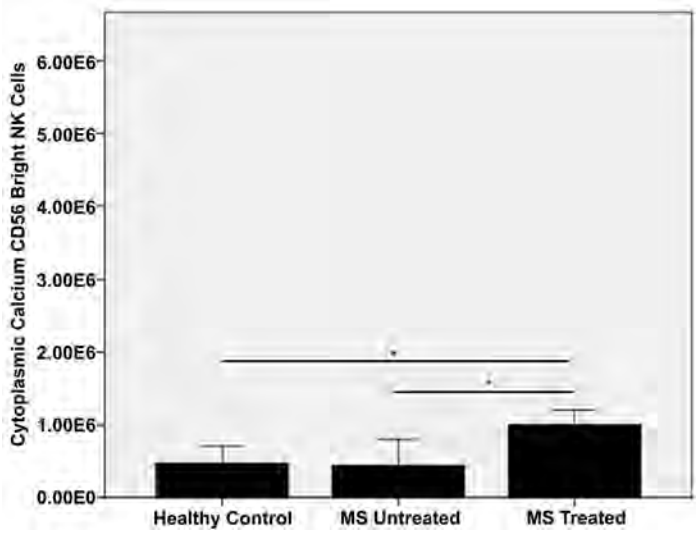


(b)

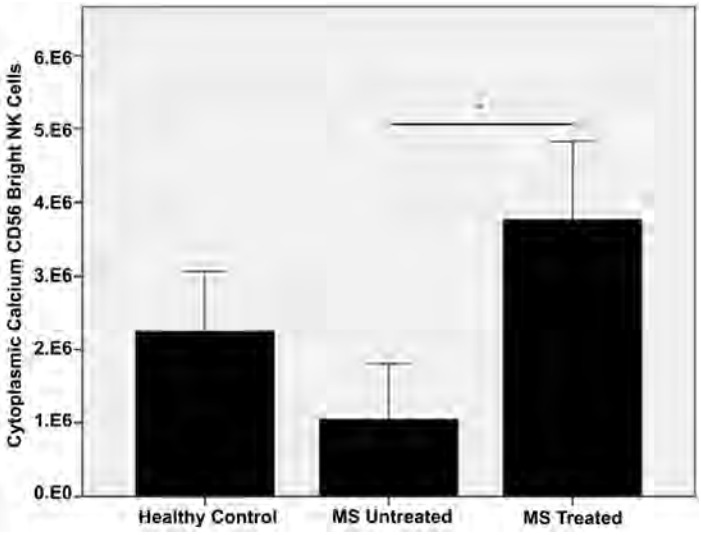
Figure 4. CD107a expression on NK cells. (a) CD107a expression on $CD56^{Bright}$ NK cells; (b) CD107a expression on $CD56^{Dim}$ NK cells. Data are represented as mean ± SEM. * represent statistical significance at p value < 0.05. Abbreviations: NK, natural killer; MS, multiple sclerosis.

2-ABP (**Figure 5(d)**) treatment (p values 0.019 and 0.004 respectively). Under treatment with PregS alone, there was a trend towards increased AUC in the treated MS group compared with HC that did not reach significance (p value 0.071) (**Figure 5(c)**). The $CD56^{Bright}$ NK cell population showed significantly increased AUC in the treated MS group compared with the untreated MS group under all treatment conditions except PregS + 2-ABP (**Figure 5(d)**).

The $CD56^{Dim}$ NK cell population showed significant increase in AUC in both the untreated and treated MS groups compared with HC when under treatment with PregS + 2-ABP (**Figure 6(a)**). There was no significant increase in AUC between subgroups under PregS or 2-ABP alone. The $CD56^{Dim}$ NK cell population


Cytoplasmic Calcium CD56 Bright NK Cells
6.00E6
5.00E6
4.00E6
3.00E6
2.00E6
1.00E6
0.00E0
Healthy Control
MS Untreated
MS Treated

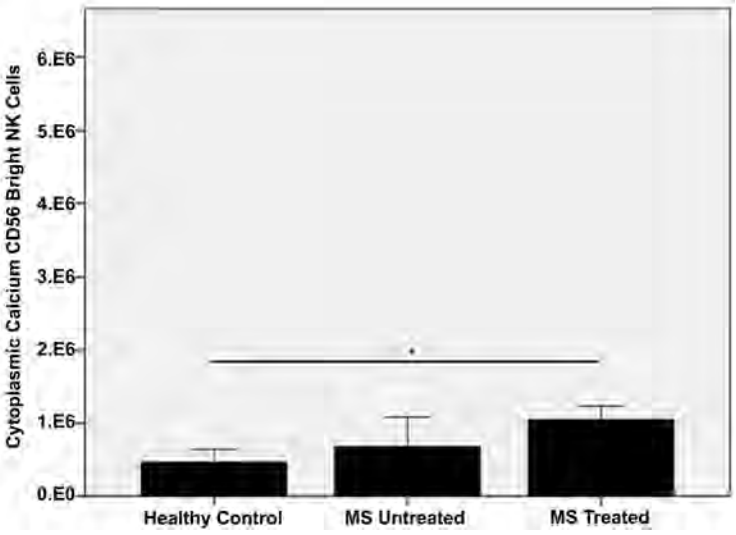
(a)


Cytoplasmic Calcium CD56 Bright NK Cells
6.E6
5.E6
4.E6
3.E6
2.E6
1.E6
0.E0
Healthy Control
MS Untreated
MS Treated

(b)


Cytoplasmic Calcium CD56 Bright NK Cells
6.E6
5.E6
4.E6
3.E6
2.E6
1.E6
0.E0
Healthy Control
MS Untreated
MS Treated

(c)


Cytoplasmic Calcium CD56 Bright NK Cells
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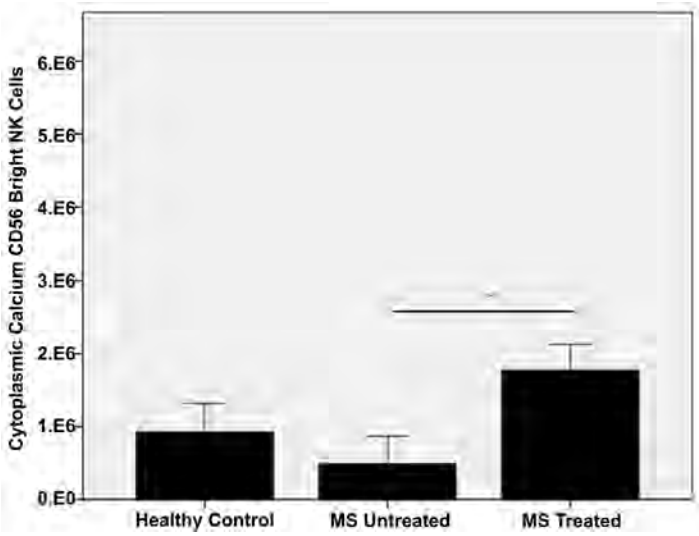
2.E6
1.E6
0.E0
Healthy Control
MS Untreated
MS Treated

(d)


Cytoplasmic Calcium CD56 Bright NK Cells
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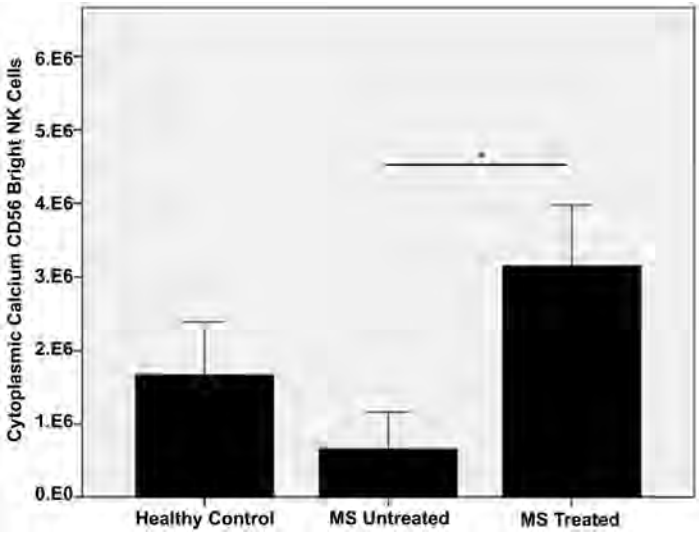
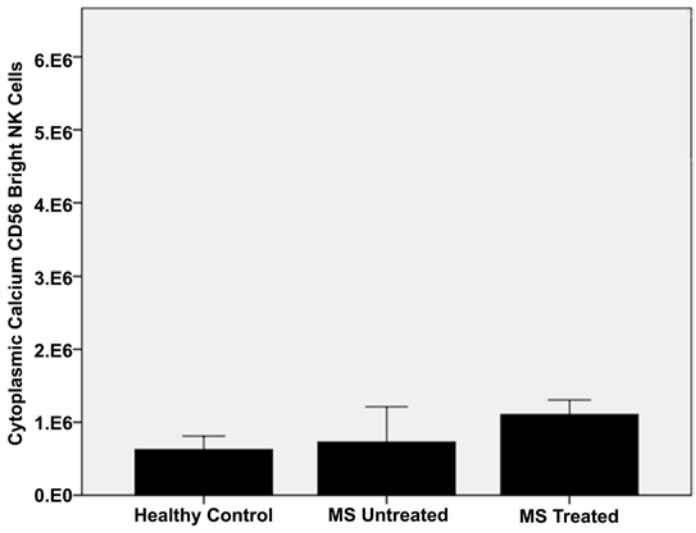
4.E6
3.E6
2.E6
1.E6
0.E0
Healthy Control
MS Untreated
MS Treated

(e)


Cytoplasmic Calcium CD56 Bright NK Cells
6.E6
5.E6
4.E6
3.E6
2.E6
1.E6
0.E0
Healthy Control
MS Untreated
MS Treated

(f)



(g)

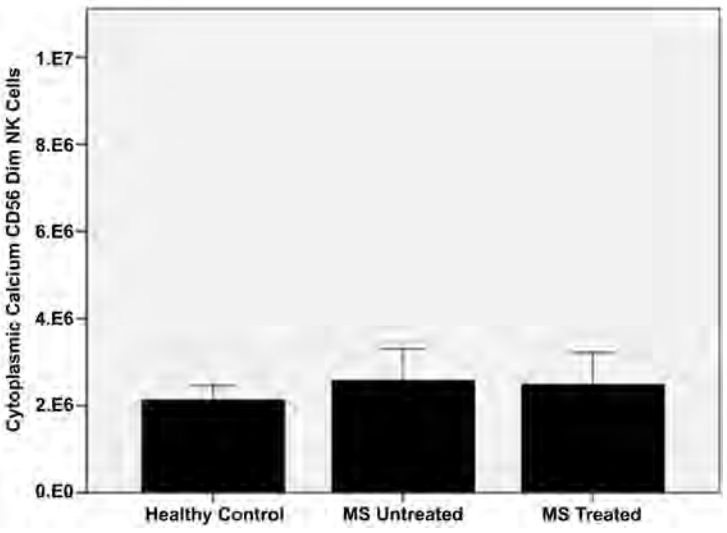
Figure 5. Cytoplasmic calcium in $CD56^{Bright}$ NK cells. (a) Ca^{2+} flux response area under the curve-treated 2-ABP; (b) Ca^{2+} flux response area under the curve-treated Ionomycin. (c) Ca^{2+}flux response area under the curve-treated PregS; (d) Ca^{2+} flux response area under the curve-treated PregS + 2-ABP; (e) Ca^{2+} flux response area under the curve-treated PregS + TG. (f) Ca^{2+} flux response area under the curve-treated TG. (g) Ca^{2+} flux response area under the curve-US. Data are represented as mean ± SEM. Asterisks (*) represent statistical significance at p value < 0.05. Abbreviations: US: Unstimulated, PregS: Pregnenolone sulphate, TG: Thapsigargin, 2-ABP: 2-Aminoethoxydiphenyl borate, AUC: area under curve, MS: Multiple sclerosis, NK: natural killer.

showed significant increase in AUC in the treated MS group compared to the untreated MS group when under treatment with TG (**Figure 6(b)**).

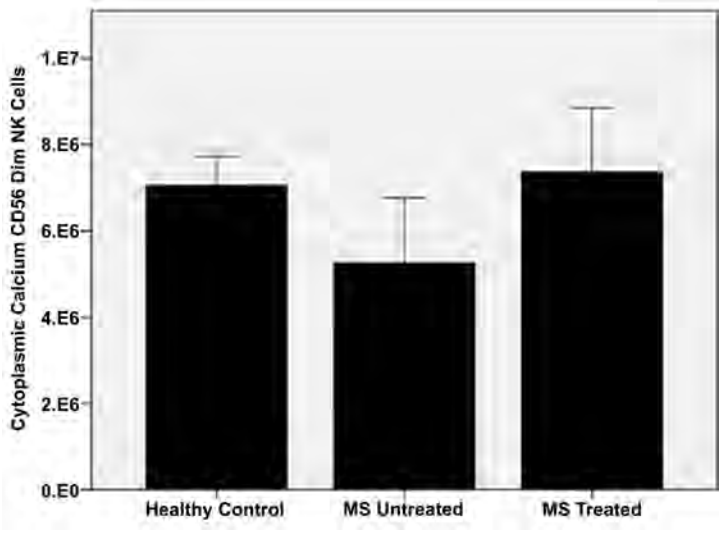
4. Discussion

This is the first study to investigate NK cell Ca^{2+} mobilisation and surface expression of TRPM3 in MS patients and HC. The current study reports no significant difference in NK cell cytotoxic activity between MS patients and HC. Importantly, variations in TRPM3, CD69 and CD107a expression were reported. Moreover, there was a significant increase in intracellular Ca^{2+} signalling in NK cells isolated from treated MS patients compared with untreated patients.

Previous investigations have reported a significant reduction in NK cell cytotoxicity against the human K562 cell line [6] [10] [11] [12] [13], conversely others have reported no difference in NK cell cytotoxic activity between MS patients and HC [43] [44]. The current investigation reported no significant changes in NK cell cytotoxicity in MS patients compared with HC. However, this investigation is limited through its small sample size. A rationale for the outcomes reported in previous investigations may be due to heterogeneity between studies with respect to study design and cohort selection. For example, flow cytometric techniques for measuring cytotoxicity are more sensitive that the chromium (Cr)-51 release assay. In addition, research suggests that NK cell cytotoxic


Cytoplasmic Calcium CD56 Dim NK Cells
1.E7
8.E6
6.E6
4.E6
2.E6
0.E0
Healthy Control
MS Untreated
MS Treated

(a)


Cytoplasmic Calcium CD56 Dim NK Cells
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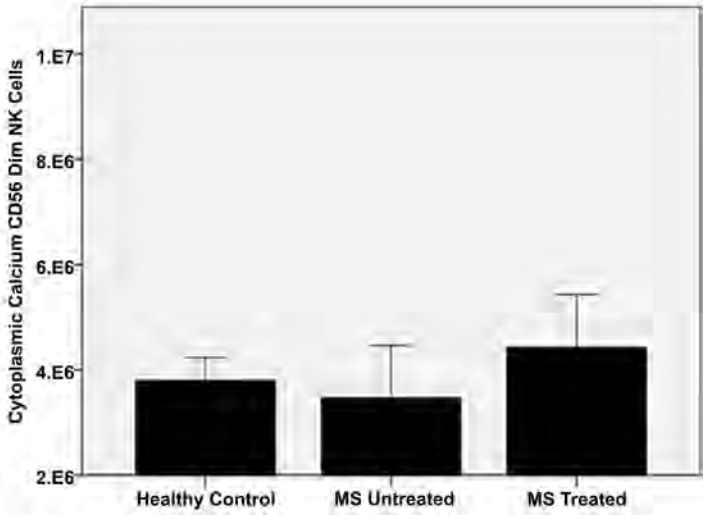
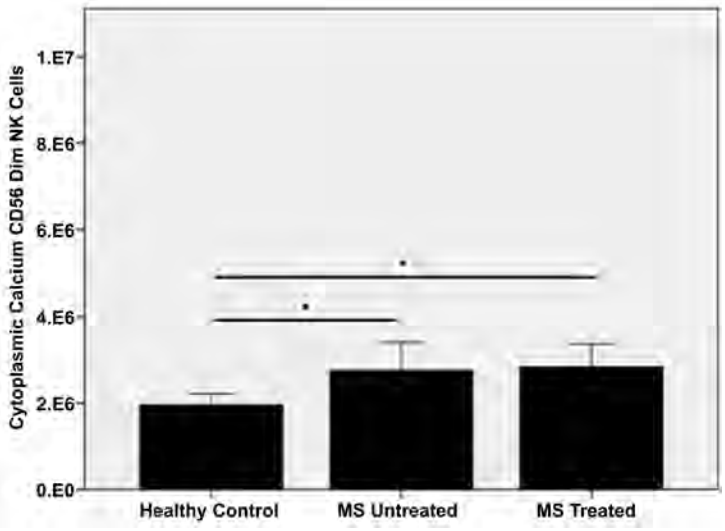
8.E6
6.E6
4.E6
2.E6
0.E0
Healthy Control
MS Untreated
MS Treated

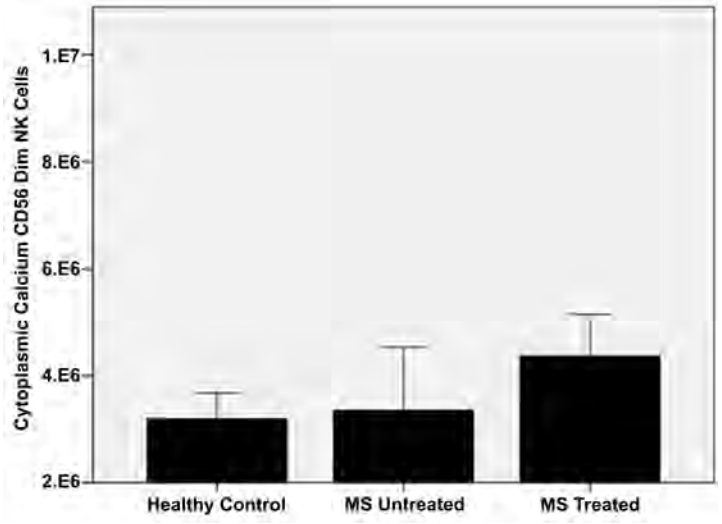
(b)


Cytoplasmic Calcium CD56 Dim NK Cells
1.E7
8.E6
6.E6
4.E6
2.E6
Healthy Control
MS Untreated
MS Treated

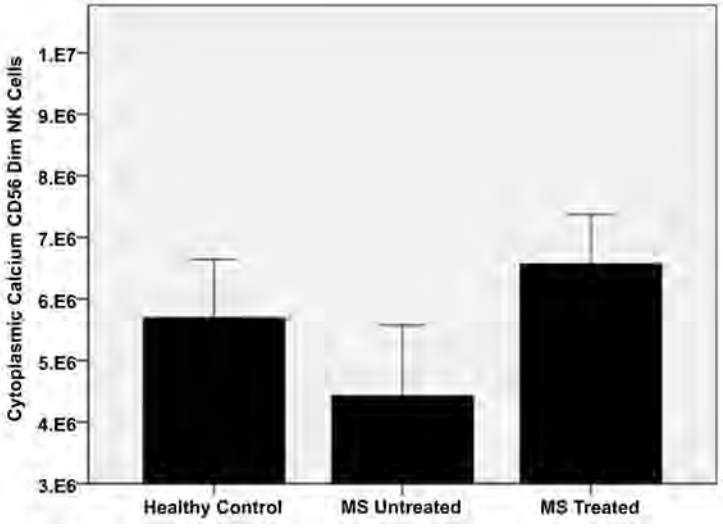
(c)


Cytoplasmic Calcium CD56 Dim NK Cells
1.E7
8.E6
6.E6
4.E6
2.E6
0.E0
Healthy Control
MS Untreated
MS Treated

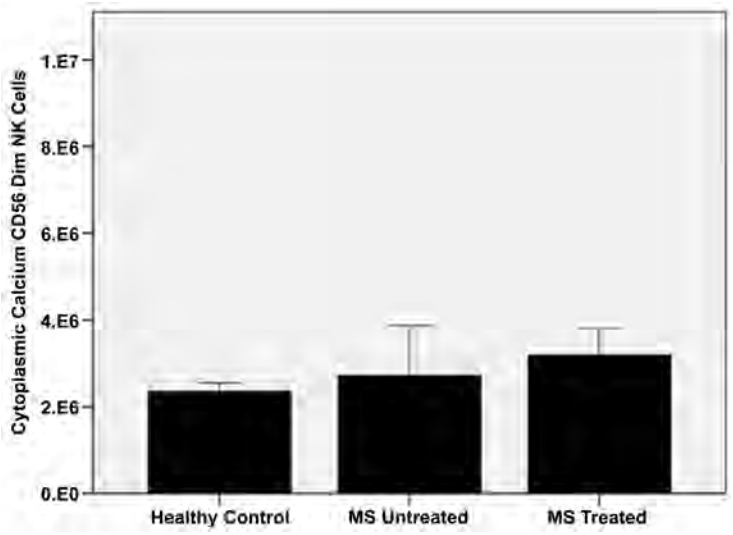
(d)


Cytoplasmic Calcium CD56 Dim NK Cells
1.E7
8.E6
6.E6
4.E6
2.E6
Healthy Control
MS Untreated
MS Treated

(e)


Cytoplasmic Calcium CD56 Dim NK Cells
1.E7
9.E6
8.E6
7.E6
6.E6
5.E6
4.E6
3.E6
Healthy Control
MS Untreated
MS Treated

(f)



(g)

Figure 6. Cytoplasmic calcium in $CD56^{Dim}$ NK cells. (a) Ca^{2+} flux response area under the curve-treated 2-ABP; (b) Ca^{2+} flux response area under the curve-treated Ionomycin. (c) Ca^{2+} flux response area under the curve-treated PregS; (d) Ca^{2+}flux response area under the curve-treated PregS + 2-ABP; (e) Ca^{2+} flux response area under the curve-treated PregS + TG; (f) Ca^{2+} flux response area under the curve-treated TG; (g) Ca^{2+} flux response area under the curve-US. Data are represented as mean ± SEM. Asterisks (*) represent statistical significance at p value < 0.05. Abbreviations: US: Unstimulated, PregS: Pregnenolone sulphate, TG: Thapsigargin, 2-ABP: 2-Aminoethoxydiphenyl borate, AUC: area under curve, MS: Multiple sclerosis, NK: natural killer.

activity is dependent on disease activity, as NK cell function is impaired during clinical relapse however normalized during remission. Therefore, our findings may reflect an immune profile of MS patients during remittent phase of disease. It is important to note alemtuzumab is believed to have little to no effect on CD52 expression on NK cells [37]. Hu *et al.* suggested that due to minimal expression of CD52, NK cells are unaffected by alemtuzumab and can continue carrying on cytotoxicity and production of cytokines [37]. As the mechanism of alemtuzumab may be a result of antibody-dependent cell mediated cytotoxicity future research will investigate CD16 expression on NK cells.

Despite not reporting significant differences in NK cell cytotoxic activity, this current investigation reports a variations in NK cell activation marker CD69 in $CD56^{Dim}$ and $CD56^{Bright}$ NK cells. CD69 is implicated in the non-selective early activation of NK cells resulting in cellular proliferation, secretion and cytotoxic activity [45]. We report that CD69 expression is significantly lower on $CD56^{Dim}$ NK cells in untreated MS patients prior to drug stimulation and following stimulation with PregS, PregS + 2-ABP and 2-ABP only. Moreover, CD69 expression is significantly increased in treated MS patients compared with untreated MS patients on $CD56^{Dim}$ NK cells.

Whilst this current investigation did not report a concurrent increase in CD69 expression $CD56^{Bright}$ NK cells, there was an upward trend in untreated MS patients compared with HC prior to drug stimulation. The reason for this is un-

known, however may be attributed to lack of NK cell-dependent immunoregulation of autoreactive T lymphocytes in untreated MS patients. Gross *et al.* suggests that alemtuzumab administration may promote NK cell efficiency in MS patients [46] possibly explaining alterations in CD69 expression seen in this investigation.

Interestingly, there was a significant increase in CD107a expression on $CD56^{Bright}$ NK cells in untreated MS patients compared with HC and patients treated with alemtuzumab. CD107a expression increases with NK cell activation and indirectly correlates with both cytokine secretion and NK cell cytotoxicity [48]. Other investigators have postulated that T lymphocyte death is mediated by $CD56^{Bright}$ NK cells through the release of pro-inflammatory cytokines [47]. Moreover, this current project suggests that variations in $CD56^{Dim}$ NK cell activation may be implicated in altered NK cell cytotoxic activity in MS patients. Altered NK cell activation and function in MS patients may be dependent on pharmacological stimulant, phase of NK cell activation (selective vs non-selective), and disease activity (relapsing remitting vs progressive; active vs remission), as evidenced by the different expression of CD69 and CD107a observed in this study.

Moreover, previous literature has examined the role of interferon therapies on NK cell function [11] [12] and the subsequent increase in the percentage of $CD56^{Bright}$ NK cells suggesting alemtuzumab expands $CD56^{Bright}$ NK cell subset [46] [49]. A previous investigation reported that removal of NK cells inhibited alemtuzumab-induced release of cytokines and cytotoxicity [37], suggesting that treatment of MS patients with alemtuzumab results in altered NK cell profiling to reflect that seen in HC. This finding suggests that an altered NK cell profile may be implicated in the pathophysiological changes in MS, and furthermore suggests that alemtuzumab may mediate therapeutic effect via alteration in NK cell profiling.

We report novel findings as TRPM3 expression was significantly increased on $CD56^{Dim}$ NK cells in untreated MS patients compared with HC following stimulation with PregS. TRPM3 expression in treated MS patients appeared comparable with HC. TRPM3 channels are activated in response to depleted intracellular Ca^{2+} stores [32] [50] and an increase in TRPM3 expression results in an increase in cytoplasmic Ca^{2+} levels [32]. In the present investigation, MS patients treated with alemtuzumab that had normalised TRPM3 expression may or may not be associated with Ca^{2+} influx. As observed in $CD56^{Bright}$ NK cells where TRPM3 expression was increased in treated MS patients, however, was not significant for $CD56^{Dim}$ NK cells. It is speculated that MS patients may have impaired Ca^{2+} influx which is compensated by increased surface expression TRPM3. These findings suggest that alemtuzumab may increase Ca^{2+} influx that potentially alters TRPM3 expression. Currently, there is no literature reporting on the effects of alemtuzumab on TRPM3 channels and Ca^{2+} mobilisation. Moreover, PregS is a potent and reversible TRPM3 agonist [51], and therefore these results may suggest a role for TRPM3 in the altered NK cell profiling as discussed previously.

Despite this however, similar changes were not seen in the unstimulated state, or under stimulation with Preg + 2-ABP or PregS + TG. Further studies with a larger cohort would be required to elucidate whether TRPM3 may be implicated in altered NK profiling in MS.

The current investigation demonstrated that under all stimulated conditions except PregS +2-ABP and Ionomycin, there was a significant increase in intracellular Ca^{2+} signalling in $CD56^{Bright}$ NK cells of patients treated with alemtuzumab compared with untreated patients. However, there was only a concurrent significant change in intracellular Ca^{2+} signalling in the $CD56^{Dim}$ NK cell population between treated and untreated patients under the treatment with TG alone. Furthermore there was no significant difference in Ca^{2+} signalling in $CD56^{Dim}$ NK cells between MS subgroups in unstimulated conditions. This study therefore suggests that whilst there may be changes in Ca^{2+} signalling due to alemtuzumab therapy in NK cells of MS patients, intracellular Ca^{2+} signalling alone is insufficient to explain the changes in NK cell profiling seen in this study.

The results of this study are limited by the small sample size however, the cohort groups were well matched with no significant differences between groups with respect to age, disease duration, EDSS, and number of relapses. The lower age of onset in the alemtuzumab group is considered to reflect a treatment bias towards patients with this disease modifying therapy in the earlier stages of disease. Similarly, the alemtuzumab group demonstrated a significantly reduced lymphocyte count when compared with HC and untreated MS patients. This may reflect alemtuzumab-dependent elimination of lymphocytes bearing CD52 [33].

5. Conclusion

This pilot study supports previous literature in that while the overall cytotoxic function of NK cells in MS patients does not appear impaired, we demonstrate that NK cell profiling is altered in MS patients, with an upregulation of $CD56^{Dim}$ NK cell populations expressing CD69, and that these effects can be abrogated by alemtuzumab therapy. Whilst intracellular Ca^{2+} signalling does not appear to account for changes in NK cell profiling of MS patients, the present study suggests that intracellular Ca^{2+} signalling in NK cells may be implicated in the therapeutic effects of alemtuzumab. The role of TRP ion channels such as TRPM3 should be further explored for their role in the pathophysiology and potential treatment of MS.

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Data Availability

Data sharing is not applicable to this article as no datasets were generated under the Griffith University Intellectual Property policy. Supporting data of this study

is included within the article.

Ethical Approval

This study was approved by Griffith University Human Research Ethic Committee (MSC/18/13).

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Conflicts of Interest

The authors have reviewed and approved the final version of this manuscript and declare no conflict of interest in the research presented.

Author Contributions

SMG, DS and SB conceived and designed experiment and provided consumables for this current investigation. LC and TN performed experiments, and participant recruitment. LC, TN and NE performed data analysis. SJ supervised research, data analysis and the writing of this manuscript. LC and NE drafted and constructed the final manuscript.

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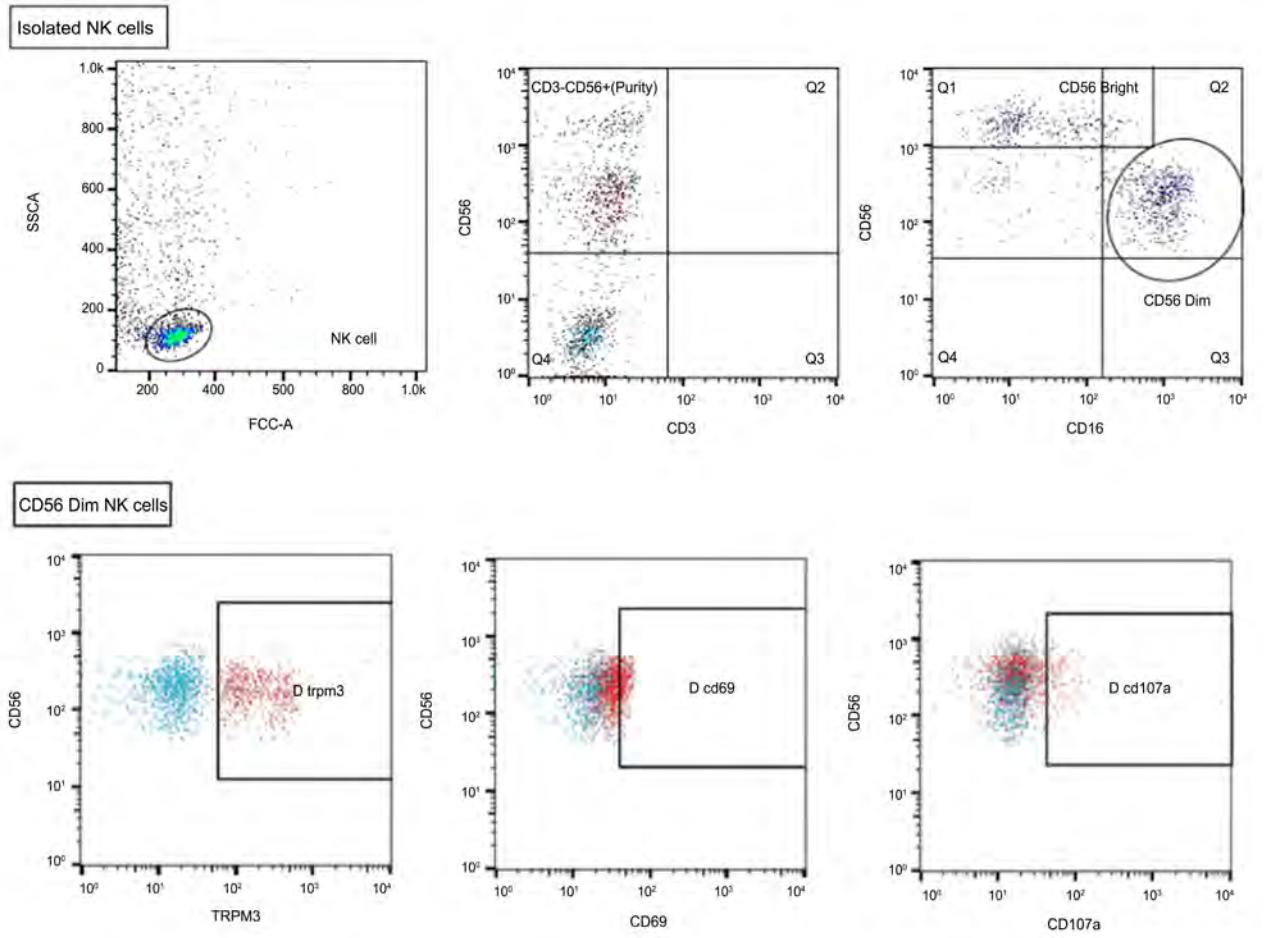
Supplementary Material



Supplementary Figure 1. Flow cytometric gating for TRPM3, CD69 and CD107a. Natural killer (NK) cells were gated ($CD56^{+}CD3^{-}$). NK cell subsets, such as $CD56^{Dim}$ and $CD56^{Bright}$ were gated using CD56 and CD16 antibodies. To determine TRPM3, CD69 and CD107a surface expression. Surface antibody staining was used and cells were gated on either $CD56^{Bright}$ or $CD56^{Dim}$ to differentiate subsets. Isotype controls were used to determine positive population.

Supplementary Table 1. Clinical and demographic data. Data shown indicates mean, SD, percentage values and p-values as indicated. Significant p-values highlighted in bold text. HC: healthy control MS: Multiple sclerosis, EDSS: expanded disability status scale, CIS: clinically isolated syndrome, RR: Relapsing remitting MS, PP: Primary progressive MS, SP: Secondary progressive MS.

Clinical Feature	HC	MS	MS-Untreated	*p*-value Untreated vs HC	MS-Treated Alemtuzumab	*p*-value treated vs HC	*p*-value untreated vs treated	*p*-value Chi squared
N	22	22	12	-	10	-	-	-
Age (years)—mean ± SD	39.4 ± 12.5	41.1 ± 15.2	46.5 ± 17.0	0.444	34.7 ± 10.0	1.0	0.138	-
Gender (female)—N (%)	6 (73)	17 (77)	8 (67)	-	9 (90)	-	-	0.426
Gender (male)—N (%)	16 (27)	5 (23)	4 (33)	-	1 (10)	-	-	
Age onset (years)—mean ± SD	-	32.6 ± 10.7	37.2 ± 10.1	-	27.2 ± 9.2	-	0.004	
EDSS—mean ± SD	-	2.4 ± 2.6	1.8 ± 2.3	-	3±2.9	-	0.434	
Total number of relapses—mean ± SD	-	3.4 ± 2.7	3.4 ± 3.3	-	3.3 ± 1.9	-	0.833	

Continued

Disease course—N (%)								
CIS	-	4 (18)	3 (25)	-	1 (10)	-	-	
RR	-	17 (77)	8 (67)	-	9 (90)	-	-	0.388
SP	-	0 (0)	0 (0)	-	0 (0)	-	-	
PP	-	1 (5)	1 (8)	-	0 (0)	-	-	

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Comparative Evaluation of Ozone Treatment in Critical Size Bone Defects Reconstructed with Alloplastic Bone Grafts

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Abstract

The purpose of this study was to investigate osteogenesis promoted by osteoconductive properties of bone grafting materials and the histopathological effects of ozone on osteogenesis. In total, 56 Winstar rats were equally divided into 4 groups. In control group, calvarial bone defect was created in 14 rats. For second group, 8 mm calvarial bone defect with ozone treatment was applied in 14 rats. For third group, an alloplastic bone graft was implanted on 8 mm calvarial bone defect. In fourth group, alloplastic bone graft was inserted in calvarial defect and ozone was treated additionally. Seven of the rats were sacrificed at the end of 4th week and the remaining 7 were sacrificed at the end of 8th week of experiment. In the study, the periosteal flaps were removed with a thin periosteal elevator and averagely 0.8 cm diameter-circular full bone defect was created with a specially designed trephine drill. The bone from the calvarial region was fixed in 10% formalin solution. After decalcification, bones were taken for routine paraffin blocking. Sections were stained with Hematoxylin-Eosin and Masson Trichrome. Histopathological findings of 4th and 8th weeks rats showed that best result for new bone formation was observed in graft + ozone treatment. It is concluded that ozone treatment increases the hemostasis in graft region, induces angiogenesis, promotes cell proliferation by preventing infiltration, induces matrix formation by influencing osteoblastic activity and has a positive effect in osteogenesis.

Keywords

Calvarial Bone Graft, Ozone, Osteoblast Cells, Rat

1. Introduction

Fracture healing depends on many actors: Local factors such as the degree of trauma, the position of the wound edges relative to each other, the vascularization of the wound area, the type of bone, the degree of immobilization, the amount of local necrosis and soft tissue damage, the presence of infections and systemic diseases (*i.e.* age, diabetes, anemia, tuberculosis); hormones such as parathyroid, calcitonin, insulin, growth hormones; vitamins such as A, C and D and general factors such as chondroitin sulphate and exercises [1] [2]. Cortical grafts provide a durable and rigid structure but they have no ability to increase osteogenesis. The primary advantage of cancellous bone and bone marrow is that they are able to significantly enhance osteogenesis. These abilities depend on the fact that they have viable cells that can transform into osteoblasts as well as those that induce osteogenesis. The only known disadvantage of these grafts is that they can't provide mechanical stability [3] [4].

Allografts are bone tissues obtained from genetically different individuals but sharing properties of same species with donor. Fresh frozen bone can be classified as frozen dried bone and demineralized bone matrix [5] [6]. Due to the limited availability of autografts, undesirable features of allografts and xenografts such as the risk of disease transfer, researchers have now been focused on synthetically graft materials which of them have been produced for use in bone defects. Since large numbers of those materials are produced, they should be studied well in researches. Alloplasts have become a necessary material in recent years for the repair of maxillofacial skeleton [7] [8].

Ozone is available in the stratosphere layer of earth at a concentration of 1 to 10 ppm in the gas phase. The disinfection property of ozone is due to its strong oxidizing property. Not only do they kill viruses and bacteria, they can also oxidize all microorganisms and their toxins. Ozone can also effectively neutralize phenols, pesticides, detergents, chemical wastes, and aromatic compounds [9]. Ozone is widely used in stomatology as a disinfectant. Besides, it also can be used in wound healing after tooth extraction, the treatment of oral infections, cases of candidiasis or periodontitis, stomatitis, treatment of infected wounds in soft tissues, and oral cavity washing prior to surgery [10]. Ozone treatments have positive effects in studies of on wound healing, ischemic and infectious diseases. It is also effectively used in many infectious diseases from simple dental and oral infections to hepatitis [11]. Studies show that ozone treatment has benefited significantly in pit and fissure caries [12], non-cavitated occlusal caries [13], and root caries at the initial stage [12] [13] [14]. The aim of this study was to investigate the histopathological effects of ozone on bone graft materials and to ex-

amine osteogenesis development induced by osteoconductive properties of these materials.

2. Materials and Methods

This study was carried out at the Dicle University Health Sciences and Application Center (DUSAM) with the approval of the Dicle University Experimental Animal Ethics Committee with the protocol dated 12.11.2013 and numbered 2013/55. In the study, 56 male Wistar rats weighing 280 - 300 grams were used and housed individually in suitable cages at temperatures of 22˚C ± 2˚C and 12 hours of dark/12 hours of light conditions. The nutritional needs of the animals were met regularly with standard laboratory food and water. Four groups (14 rats per group) were arranged as below:

1) Control Group: Calvarial bone defect was created in 14 rats without any additional treatment and wound was sutured. Seven of the subjects were sacrificed at the end of the 4th week, and the remaining 7 were sacrificed at the end of the 8th week.

2) Experimental Group: 8 mm calvarial bone defects were created in all rats and then treated with ozone. 7 of the subjects were sacrificed at the end of the 4th week, and the remaining 7 were sacrificed at the end of the 8th week.

3) Experimental Group: 8 mm calvarial bone defects were created in all rats and then alloplastic bone grafts were applied to the defect. 7 of rats were sacrificed at the end of the 4th week and the remaining were sacrificed at the end of the 8th week.

4) Experimental Group: 8 mm calvarial bone defects were created in all rats and then alloplastic bone grafts + ozone treatment was applied to the defect. Seven of them were sacrificed at the end of the 4th week, and the remaining were sacrificed at the end of the 8th week.

2.1. Surgical Operation

The animals were anesthetized with intraperitoneally 3 mg/kg xylazine (Rompun 2%; Bayer) and 90 mg/kg Ketamine HCl (Ketalar; Eczacıbası-Warner Lambert). Skin was incised to open frontal bone. A periosteal flap was removed with a thin elevator. Surgical sites were exposed with an incision through the skin and the periosteum at the midline of the calvaria. The periosteal flap was removed with a thin periosteal elevator and a specially designed trephine bur was created with a circular full-thickness bone defect with a diameter of 0.8 cm on the midline. After the graft was applied in our study, topical Ozone (Prozone W & H, Bürmoos, Austria) was applied for 12 seconds with a Coro-type applicator. Allografts are bone tissues of the same species as the recipient but derived from genetically distinct individuals. It is used as fresh frozen bone, frozen dried bone and demineralized bone matrix. Allograft material was used as demineralized bone matrix in our study.

Alloplastic material (Bio-Graft-HT) was placed in defect area in group 3 and group 4. Subcutaneous tissue was sealed with 6/0 vicryl suture and skin was

closed using 5/0 silk suture. Half of the experimental animals in all groups were sacrificed at the end of the 4th week and the remainder was sacrificed at the end of the 8th week by intraperitoneally overdose of sodium thiopentone. The skin on the calvarium was completely removed and the defect was taken out with bone forceps.

2.2. Histopathological Protocol

The bones from the calvarial region were fixed in 10% formalin solution. They were decalcified for 1 week in 10% ethylene diamine tetra acetic acid (EDTA) solution. After washing, tissues were dehydrated through ascending alcohol series and cleared with xylene. After embedding them in paraffin, 4 - 6 μm thick sections were taken with Leica ultramicrotome. Sections were stained with Masson Trichrome and Hematoxylin-Eosin and examined by light microscope.

2.3. Statistical Analysis

Statistics and analyzes were performed using the SPSS 22.0 for Windows computer package program, and the results were considered statistically significant for $p < 0.05$. In the analysis of the data, Kruskall-Wallis and Mann-Whitney U non-parametric statistical tests were used in the intergroup comparisons depending on the variables and the results were given as mean and standard deviation values.

3. Results

Histopathological Findings

1) 4th week findings

a) Control Group: Figure 1(a) shows a significant increase was observed in osteoclast cells in defect area. Hemorrhage and dilatation in blood vessels and mononuclear cell infiltration were increased. Defect Group: In Figure 1(a*), in other section of the same group, an increase in fibrous tissue and collagen fibers, freely dispersed erythrocytes due to tearing in the vessel wall, intense mononuclear cell infiltration and rarely edema were observed.

b) Defect + Ozone treated Group: Sections showed dilatation was significantly increased in the veins. Comparing with the defect group, there was a decrease in fibrous tissue and osteoblastic activity in this group. In another section, the collagen fibers were bundled, the connective tissue were increased, new bone trabeculae were formed and the bone matrix began to become evident.

c) Defect + Graft Applied Group: Osteoblasts were clearly observable, connective tissue cells and fibrous structure around bone trabeculae were increased, graft resorption was decreased. A decrease in osteoblasts and cell infiltration was observed. In another section, bone trabeculae were more abundant around the veins and collagen fibers were observed in bundles. Decrease in connective tissue cells and dispersed erythrocytes were observed. A small number of osteocytes were found in bone trabeculae.

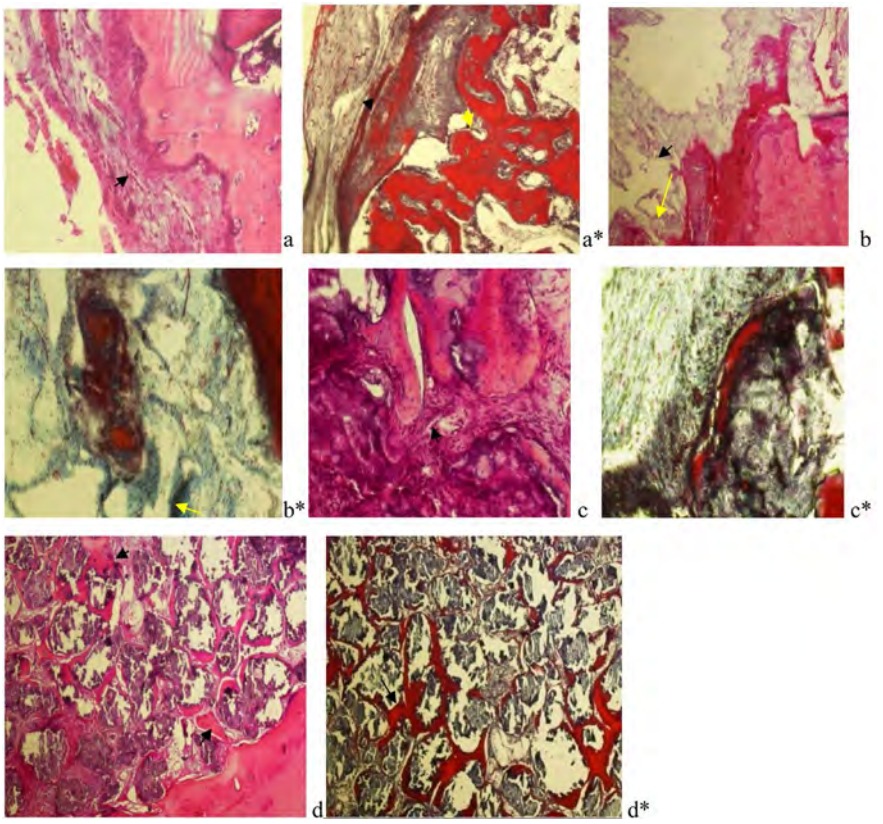

Figure 1. a: A significant increase was observed in osteoblast cells in defect area (arrow). H-E staining Bar 50 μm; a*: An increase in fibrous tissue and collagen fibers (arrow), dilatation in blood vessels (yellow arrow) Trichrome Masson staining Bar 50 μm, b-İnflamatory cells in defect area (arrow) H-E staining Bar 50 μm; b*: hemorrhage in blood vessels (yellow arrow) Trichrome Masson staining Bar 50 μm, c-osteoblast cells around bone trabecula (arrow), H-E staining Bar 50 μm; c*: intense inflammatory cells in graft area (yellow arrow) Trichrome Masson staining Bar 50 μm; d: An increase in osteoblastic activity and osteocyte cells (arrow), H-E staining Bar 50 μm; d*: Appearance of mature bone particles (arrow) Trichrome Masson staining Bar 50 μm.

d) Defect + Graft + Ozone Applied Group: In sections of this group, graft resorption began, bone trabeculae became evident due to an increase in dense connective tissue fibers and osteoblastic activity around the graft. Matrix development was increased and a small number of osteocytes in bone trabeculae were observed. In another section, dense connective tissue around the graft was enriched by especially collagen fibers, new bone formation in connective tissue began to increase and osteocyte cells with their lacunae became evident in new bone trabeculae.

For the 4th week, cell infiltration was significantly different between the groups ($p < 0.0001$). When we examined osteoclastic activity of week 4, there was a significant difference between the groups ($p = 0.0001$). There was a significant difference between the groups in osteoblastic activity of 4th week ($p = 0.253$). There was a significant difference between the groups in terms of matrix formation ($p = 0.0007$), bone trabeculae ($p = 0.0018$, vascular dilatation and hemorrhage ($p =$

0.0005) and collagen fiber distribution ($p = 0.0099$) (Table 1).

2) 8th week findings

a) Control Group: In Figure 2(a), bone trabeculae were visible and osteoblasts were flattened and arranged in a single row around the trabeculae. While cell infiltration mostly gathered around the trabeculae, edema was seen in some areas. Vessels were dilated and apparently hemorrhagic. Figure 2(a*) shows the collagen fibers were in bundled form, islets of bone trabeculae were seen, the osteoblastic activity was increased in the trabeculae and the matrix development

Table 1. Statistical evaluation of parameters according to 4th week according to different groups.

(a)

Parameter's	Groups	n	Mean	Average Rank	Kruskal-Wallis Test value	Multiple comparisons for groups ($p < 0.05$)
Cell infiltration	(1) Control group	7	3.71	22.00	20.3276; $p < 0.0001$	(3) (4)
	(2) Defect + Ozone	7	3.57	21.00		(3) (4)
	(3) Defect + Graft	7	0.00	7.50		(1) (2)
	(4) Defect + Graft + Ozone	7	0.00	7.50		(1) (2)
Osteoclastic activity	(1) Control group	7	0.00	4.00	20.1291; $p = 0.0001$	(2) (3) (4)
	(2) Defect + Ozone	7	2.71	20.07		(1) (3)
	(3) Defect + Graft	7	1.71	12.43		(1) (2) (4)
	(4) Defect + Graft + Ozone	7	3.00	21.50		(1) (3)
Osteoblastic activity	(1) Control group	7	1.57	18.93	2.9008; $p = 0.2533$	ns
	(2) Defect + Ozone	7	1.14	13.79		
	(3) Defect + Graft	7	1.00	11.93		
	(4) Defect + Graft + Ozone	7	1.14	13.36		
Matrix formation	(1) Control group	7	3.00	20.50	15.5120; $p = 0.0007$	(2) (4)
	(2) Defect + Ozone	7	2.00	11.71		(1) (3) (4)
	(3) Defect + Graft	7	3.00	20.00		(2) (4)
	(4) Defect + Graft + Ozone	7	1.29	5.79		(1) (2) (3)
Bone trabeculae	(1) Control group	7	2.14	13.36	10.6784; $p = 0.0018$	(4)
	(2) Defect + Ozone	7	1.71	8.64		(4)
	(3) Defect + Graft	7	2.14	13.36		(4)
	(4) Defect + Graft + Ozone	7	2.86	22.64		(1) (2) (3)
Vessel dilatation and hemorrhage	(1) Control group	7	2.14	16.29	15.6492; $p = 0.0005$	(3)
	(2) Defect + Ozone	7	2.29	17.36		(3)
	(3) Defect + Graft	7	0.29	4.14		(1) (2) (4)
	(4) Defect + Graft + Ozone	7	2.57	20.21		(3)
Collagen fibers	(1) Control group	7	2.71	17.79	9.0654; $p = 0.0099$	(3)
	(2) Defect + Ozone	7	2.71	17.79		(3)
	(3) Defect + Graft	7	1.71	6.50		(1) (2) (4)
	(4) Defect + Graft + Ozone	7	2.57	15.93		(3)

ns: non-significant; $p > 0$ 05.

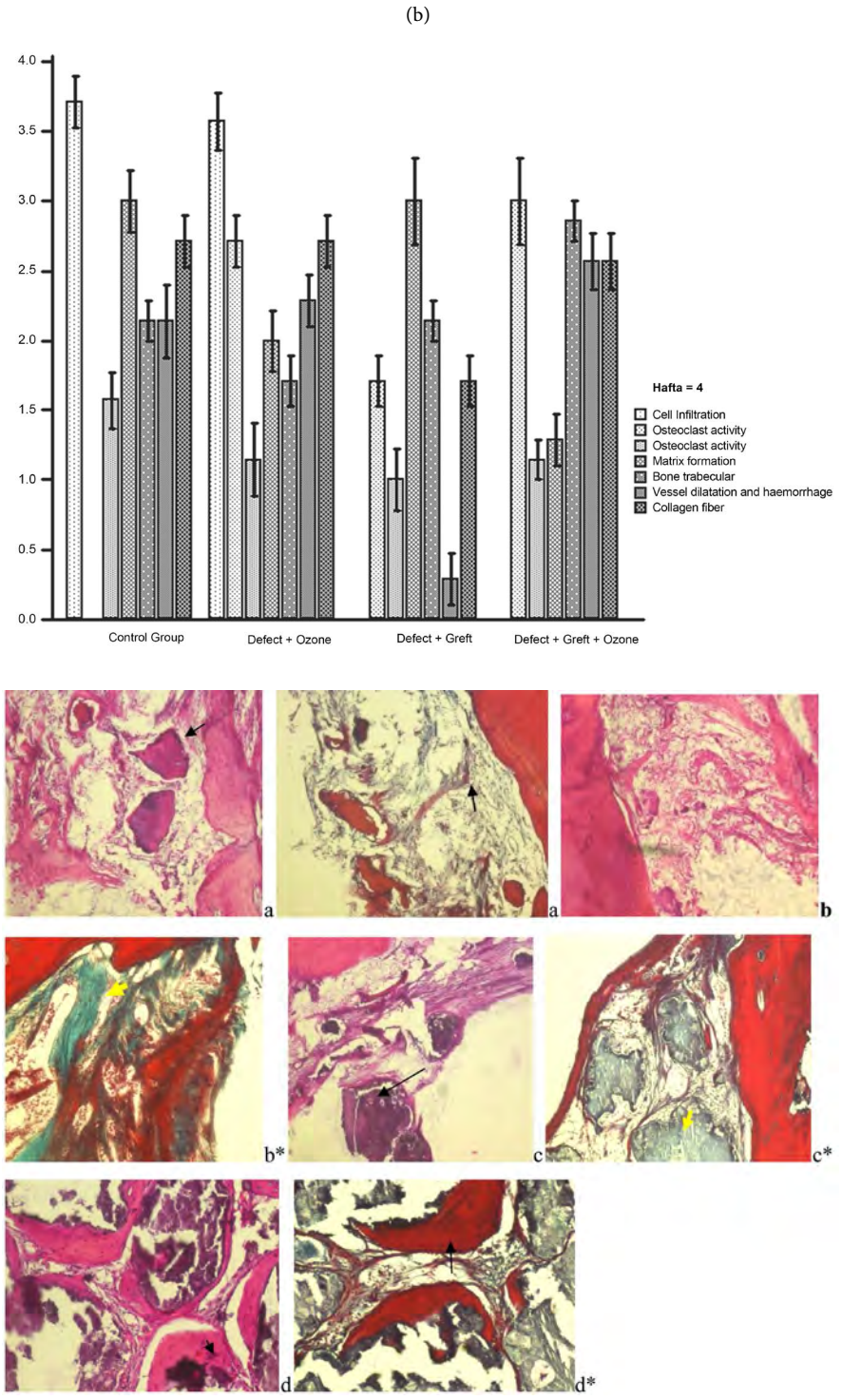

Figure 2. a: bone trabeculae in defect area (arrow). H-E staining Bar 50 μm; a*: An increase in fibrous tissue and collagen fibers(arrow), Trichrome Masson staining Bar 50 μm; b: Inflammatory cells in defect area (arrow) H-E staining Bar 50 μm; b*: increase of collagen fibers in defect area (yellow arrow) Trichrome Masson staining Bar 50 μm; c: Appearance of mature bone trabecula (arrow), H-E staining Bar 50 μm; c*: Enlargement bone trabecula in graft area (yellow arrow) Trichrome Masson staining Bar 50 μm; d: An increase in bone trabeculae and osteocyte cells (arrow), H-E staining Bar 50 μm; d*: Appearance of mature bone particles (arrow) Trichrome Masson staining Bar 50 μm.

started. Extracellular matrix of connective tissue was increased. Form of fully developed mature bone cells were not observed in this group.

b) Defect + Ozone Applied Group: Islets of inflammatory cells; hemorrhage and dilatation in vessels of the defect areacan be seen in Figure 2(b). Throughout new bone formation, matrix development was apparently begun. There were bone trabeculae in some areas. Figure 2(b*) shows thickening in collagen fibers, intense hemorrhagic areas in defect region, enlargement of the bone matrix and new bone trabeculae formation were observed.

c) Defect + Graft Group: The histological examination of this group showed new bone trabeculae with increased connective tissue around the graft sites. As cell infiltration continued, freely distributed erythrocytes were found in connective tissue. Osteoblast cells were clearly observed around the bone trabeculae as seen in Figure 2(c). Graft resorption was dispersed and graft areas began to diminish gradually. In Figure 2(c*), collagen fibers were tightly gathered around graft areas and made resorbed grafts reduce. Inflammatory cells spread diffusely between graft areas, and new bone particles began to become apparent in the periphery of graft.

d) Defect + Graft + Ozone Applied Group: Figure 2(d) shows that the new bone trabeculae around the graft areas began to turn into mature flat bone and osteocytes cells were abundant due to increased osteoblastic activity. Cell infiltration was gradually reduced and mature bone trabeculae were formed in the site of resorbed graft. In Figure 2(d*), revealed shrinkage in the resorbed graft sites, the structures of mature bone particles became evident and fibrillary development in the connective tissue accelerated and wrapped around the bone-trabeculae.

There was a significant difference between the groups when the weekly cell infiltration ($p = 0.0014$), osteoclastic ($p = 0.0004$) and osteoblastic ($p = 0.0055$) activities were examined. Matrix formation at 8 weeks ($p = 0.0001$), Bone trabeculae ($p = 0.0002$) There was a significant difference between the groups when vascular dilatation and hemorrhage ($p < 0.0001$) and collagen fiber distribution ($p < 0.0001$) (Table 2 and Table 3).

Table 2. Statistical evaluation of parameters according to 8th week according to different groups.

(a)

Parameter	Groups	n	Mean	Average Rank	Kruskal-Wallis Test value	Multiple comparisons for groups ($p < 0\ 05$)
Cell infiltration	(1) Control	7	3.29	22.50	13.3723; $p = 0.0014$	(3) (4)
	(2) Defect + Ozone	7	2.86	17.21		(3) (4)
	(3) Defect + Graft	7	2.86	8.64		(1) (2)
	(4) Defect + Graft + Ozone	7	1.14	9.64		(1) (2)
Osteoclastic activity	(1) Control	7	2.71	6.57	14.5683; $p = 0.0004$	(2) (4)
	(2) Defect + Ozone	7	1.29	21.57		(1) (3)

Continued

	(3) Defect + Graft	7	1.00	11.21		(2) (4)
	(4) Defect + Graft + Ozone	7	0.00	18.64		(1) (3)
Osteoblastic activity	(1) Control	7	1.86	6.14	9.6334; $p = 0.0055$	(2) (3) (4)
	(2) Defect + Ozone	7	2.29	17.29		(1)
	(3) Defect + Graft	7	2.86	17.29		(1)
	(4) Defect + Graft + Ozone	7	4.00	17.29		(1)
Matrix formation	(1) Control	7	2.00	20.43	17.8543; $p = 0.0001$	(2) (4)
	(2) Defect + Ozone	7	2.29	12.57		(1) (3) (4)
	(3) Defect + Graft	7	2.14	20.43		(2) (4)
	(4) Defect + Graft + Ozone	7	4.00	4.57		(1) (2) (3)
Bone trabeculae	(1) Control	7	1.86	19.14	16.0978; $p = 0.0002$	(2) (4)
	(2) Defect + Ozone	7	2.29	9.86		(1) (3)
	(3) Defect + Graft	7	3.14	22.00		(2) (4)
	(4) Defect + Graft + Ozone	7	3.57	7.00		(1) (3)
Vessel dilatation and hemorrhage	(1) Control	7	3.14	17.57	24.7621; $p < 0.0001$	(2) (3) (4)
	(2) Defect + Ozone	7	2.86	11.43		(1) (3) (4)
	(3) Defect + Graft	7	1.86	4.00		(1) (2) (4)
	(4) Defect + Graft + Ozone	7	1.29	25.00		(1) (2) (3)
Collagen fibers	(1) Control	7	2.29	23.00	20.9831; $p < 0.0001$	(2) (3) (4)
	(2) Defect + Ozone	7	2.14	18.71		(1) (3) (4)
	(3) Defect + Graft	7	2.14	4.14		(1) (2) (4)
	(4) Defect + Graft + Ozone	7	2.86	12.14		(1) (2) (3)

ns: non-significant; $p > 0.05$.

(b)

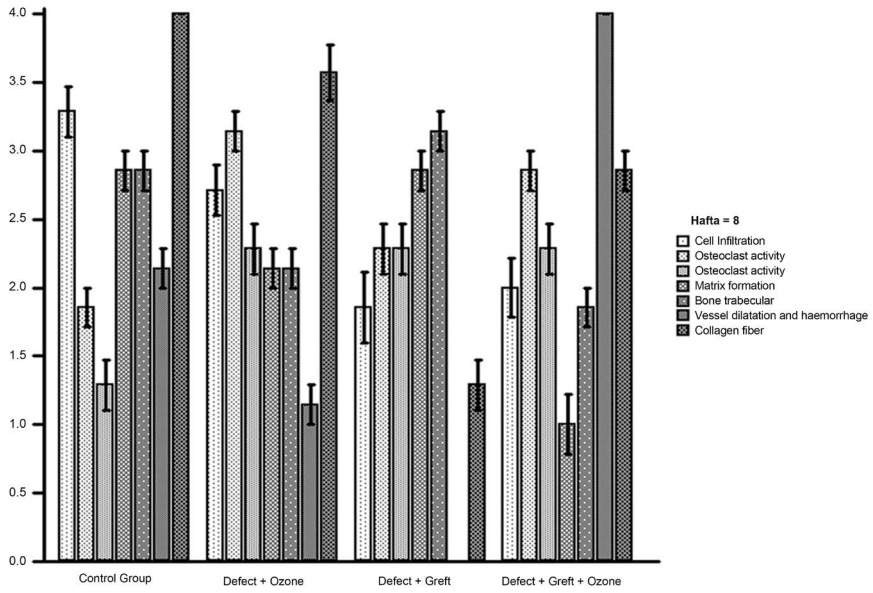

Table 3. Statistical evaluation of parameters according to 4th and 8th week according to different groups.

Parameters	Groups	Weeks	Mean	Mean Rank	*p*
Cell infiltration	Control group	4	3.71	1.71	0.180
		8	3.29	1.29	
	Defect + Ozone	4	3.00	1.57	0.655
		8	2.86	1.43	
	Defect + Graft	4	3.00	1.57	0.655
		8	2.86	1.43	
	Defect + Graft + Ozone	4	2.29	2.00	0.008
		8	1.14	1.00	
Osteoclastic activity	Control group	4	3.57	1.93	0.014
		8	2.71	1.07	
	Defect + Ozone	4	1.57	1.64	0.317
		8	1.29	1.36	
	Defect + Graft	4	1.29	1.57	0.317
		8	1.00	1.43	
	Defect + Graft + Ozone	4	0.29	1.64	0.157
		8	0.00	1.36	
Osteoblastic activity	Control group	4	0.00	1.00	0.008
		8	1.86	2.00	
	Defect + Ozone	4	1.14	1.07	0.014
		8	2.29	1.93	
	Defect + Graft	4	2.14	1.14	0.025
		8	2.86	1.86	
	Defect + Graft + Ozone	4	2.57	1.00	0.008
		8	4.00	2.00	
matrix formation	Control group	4	.00	1.00	0.008
		8	2.00	2.00	
	Defect + Ozone	4	1.00	1.00	0.008
		8	2.29	2.00	
	Defect + Graft	4	1.71	1.29	0.083
		8	2.14	1.71	
	Defect + Graft + Ozone	4	2.71	1.00	0.008
		8	4.00	2.00	
Bone trabeculae	Control group	4	0.00	1.00	0.008
		8	1.86	2.00	
	Defect + Ozone	4	1.14	1.07	0.014
		8	2.29	1.93	
	Defect + Graft	4	2.14	1.07	0.014
		8	3.14	1.93	
	Defect + Graft + Ozone	4	2.71	1.14	0.025
		8	3.57	1.86	

Continued

Vessel dilatation and hemorrhage	Control group	4	2.71	1.29	0.083
		8	3.14	1.71	
	Defect + Ozone	4	3.00	1.57	0.564
		8	2.86	1.43	
	Defect + Graft	4	2.86	1.93	0.014
		8	1.86	1.07	
	Defect + Graft + Ozone	4	1.71	1.71	0.083
		8	1.29	1.29	
Collagen fibers	Control group	4	1.71	1.21	0.046
		8	2.29	1.79	
	Defect + Ozone	4	2.00	1.43	0.564
		8	2.14	1.57	
	Defect + Graft	4	2.14	1.50	1.000
		8	2.14	1.50	
	Defect + Graft + Ozone	4	2.57	1.36	0.317
		8	2.86	1.64	

When compared histologically at 4 and 8 weeks; colloid fiber growth, matrix formation and new bone formation were less than 4 weeks to 8 weeks. Vessel dilatation and hemorrhage increased cell infiltration over 4 weeks to 8 weeks, osteoblastic activity significantly increased bone trabecular formation at 8 weeks. In bone formation, post-defect graft application was evident at 8 weeks, ozone application increased bone formation as an inducing agent.

4. Discussion

Nagayoshi *et al.* [15] found that no microorganisms remained in the plaque after ozonized water treatment on the dental plaque. In oral surgery, ozonized water is used to provide hemostasis, to increase local oxygen delivery to the site, and to inhibit bacterial proliferation [13]. Ozonized water has also been proposed to use as a prophylactic agent against infection after osteotomy during oral surgery. Following the high dose of radiotherapy treatment, ozone treatment has been shown to be effective in improvement of wound healing [16]. After radiotherapy in maxilla or mandible, in areas where especially oxygen is greatly reduced, spongy medullary regions become inadequately vascularized due to deterioration of vascularity, resulting in aseptic osteonecrosis [16]. In surgical procedures such as tooth extraction or implant application, the healing of the damaged region takes longer than the healing of the healthy bone [16] [17]. Schmitz and his colleagues studied in 3, 4, 5 and 8 mm defects and suggest that an 8 mm defect is an ideal defect [18]. In our study, 8 mm defects were created. Different bone graft materials have been used for bone regeneration, closure of osteotomy openings, and alveolar augmentation in oral and maxillofacial surgeons [19]

[20]. Natural coral-derived grafts, synthetic bone graft materials, are used in alveolar crest elevation, intra-bone defects, material loss fractures, facial bone defects, orthognathic surgery, and maxillary sinus ground [20] [21] [22]. In our study, an alloplastic graft material consisting of a combination of 350 - 500 μm-diameter porous biphasic hydroxyapatite granules and β-tricalcium phosphate granules was used. Agrillo *et al.* have studied the effects of ozone on wound healing after tooth extraction in 15 patients using bisphosphonates. Topical ozone gas with β-lactam antibiotics were applied to all patients 1 week before and after tooth extraction. Clinical and radiological follow-ups of patients showed complete healing in the extraction socket [23]. Kazancıoğlu *et al.* formed a 5 mm diameter bone defect in 30 Wistar rats. They created defects and filled them with biphasic calcium phosphate grafts. They categorized rats into three groups and histomorphometrically compared the effect of laser and ozone on new bone formation. Kazancıoğlu *et al.* observed significant bone formation in both groups compared to the control group [24]. Özdemir *et al.* have studied the effect of ozone treatment on autogenous bone graft healing in calvarial defects. Histological results of this study revealed that there was an increase in osteoblastic activity and significant difference in bone formation in ozone treated group [25]. In the histopathological examination of week 4 of our study, a significant increase in osteoblastic activity was observed in all compared to the control group. Week 8 results showed significant increase in osteoblastic activity in graft + ozone group compared to all other groups. More bone trabecular formation was observed in the groups treated with grafts and grafts + ozone compared to the group treated with only ozone. 4th week histological evaluations on matrix formation showed a significant increase in all groups compared with the control group at the end of. Statistically speaking on histological results, 4-week ozone treatment is less effective than 8-week ozone treatment and additionally 8-week treatment is more effective and significant than 4-week-treatment in total. In our study, there was a significant difference between the groups treated with s graft + ozone and the other groups in terms of cell infiltration in the groups sacrificed at 8 weeks. There was no significant difference in comparison between the other groups. Erdemci *et al.* studied on the effect of systemic ozone on alveolar bone healing. A total of 112 Wistar rats were used, and they were sacrificed on days 14 and 28. They reported systemic ozone treatment significantly improved alveolar bone healing after extraction [26]. In their study, unlike the half of the rats at the end of 4 week, the remaining half was sacrificed at the end of the 8th week. Each group was divided into 2 subgroups. A critical size bone defect of 8 mm in diameter was formed in the calvarium of all rats. Topical ozone was applied to the ozone treated groups for 12 sec.

In our study, it was determined that ozone application accelerated bone healing in parallel with this study. It was observed that topical ozone administration at week 4 failed to exert only osteoblastic activity on graft application. However, ozone and graft combination may be beneficial. When the bone trabeculae were

taken to evaluate the 8th week groups, a statistically significant increase was observed only in the graft and graft + ozone treated groups compared to the control group When the 4- and 8-week findings were evaluated; the best result for new bone formation was obtained from the graft + ozone treated group. It is thought that ozone application increases the hemostasis in the region and induces angiogenesis, increases cell proliferation by preventing infiltration. It also promotes osteoblastic activity and matrix formation and has a positive effect on estrogen

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Vertical Ridge Augmentation of Atrophic Anterior Mandible with Autogenic Tibia Grafts Compared Allogenic Grafts

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Abstract

Aim: This study was performed to evaluate the Movement of the proximal segment following different methods of ramus osteotomy which was one of the side effects of orthognathic surgery. Theoretically, with intraoral verticosagittal ramus osteotomy, it can minimize the movement of the proximal segment. The changes in the intergonal distance of mandible and the angle of the ramus flaring in two methods of osteotomy have been compared in this study. **Materials and Methods:** This randomized clinical trial included 60 patients (32 males) with mandibular prognathism and without any asymmetry were selected and divided into two groups (n = 30). One group underwent bilateral sagittal split ramus osteotomy technique to achieve mandibular setback and the other by the intraoral verticosagittal ramus osteotomy technique. Intergonial width and inner-ramal angle in the transverse plane were measured on radiographs preoperatively and 1 and 12 weeks postoperatively. Data were analyzed using covariance test with the significance level set at $P < 0.05$. **Results:** Changes of intergonial width and inter-ramal angle were significant in both the 1 and 12-week radiographies taken post-operative in both groups. but no statistically significant difference was observed in the intergonial width and ramus flaring at the mentioned time points between the two groups ($P > 0.1$). **Conclusion:** Considering our findings there was no significant difference between two ramus osteotomy techniques regarding changes in mandibular width and inter-ramal flaring angle.

Keywords

Ridge, Ridge Augmentation, Autografting, Tibia, Osteotomy

1. Introduction

Numerous advancements regarding the application of new materials in surgery, in order to increase the under prosthesis surface have led to the greater usage of such materials in surgical interventions for ridge height augmentation. Among such materials, hydroxyapatite and allogens are worth mentioning.

Among the most well-known and oldest materials bone grafts (iliac or ribs) can be named [1] [2]. The most common sources for bone grafts are: autogenic bones, allogenic bones, alloplast or xenograft materials. Generally, if the bone height is less than 15 mm in the anterior segment of the lower jaw, vestibuloplasty is not practical; the ridge height must be initially augmented and then vestibuloplasty can be applied [3].

The weak points of autogenic grafts are the need for surgery in another body part and severe bone loss (especially in the iliac bone) following the grafting procedure. On the other hand, no need for second surgery in the patient and the patient's better comfort can be mentioned as its strong points [3]. In young patients with moderate atrophy of the alveolar ridge, ridge extension with developed vestibular surface of the jaw or vestibuloplasty seems to be a suitable technique. However, in elderly patients with severe alveolar ridge atrophy, ridge augmentation is more suitable [4].

Autogenic grafts on the mandible are most commonly used when severe bone loss has resulted in reduced bone height and width with a great risk of bone fracture. Moreover, this method is used when adequate bone is not available for placing the dental implant in the bone. However, some studies believe that autogenic grafts have more major complications and therefore allogenic grafts are a suitable alternative for autogenic grafts with fewer complications [5]. The majority of complications caused by autogenic or allogenic grafts include infection following grafting or graft rejection. Moreover, 17% of patients with a residual bone height of <6 mm have experienced graft rejection in the first 3 years [6].

The available literature have mainly focused on ridge augmentation in the maxillary bone and few studies have been performed on the adequacy of these materials in bone grafts for height augmentation of the mandible (especially when the tibia as a donor site). Therefore in this study we aimed at investigating the changes in the height of the ridge following autogenic (tibia graft) and allogenic grafts in the anterior segment of the mandible in a 6-month period, so that a more comprehensive evaluation could be given on the quality of the conducted surgeries.

2. Materials and Methods

Our study was a randomized blinded clinical trial performed in the Implant Unit of the Dentistry School of Tehran University of Medical Sciences from 2014 to 2016. The sampling method was random sampling using quadruple blocks. The inclusion criteria were systemically healthy patients (ASA l) and who were no

physical deformities. Height of anterior segment of mandible was between 10 - 14 millimeter (mean 12 millimeter). All patients signed the consent form.

Before study initiation, an informed consent was obtained from all participants and the need for treatment follow up for 6 months was described to all patients.

After patients' selection, they were randomly divided into two groups; autogenic tibia grafts were used in group A and allogenic grafts were used in group B. The selected cases were 8 males and 10 females with the mean age of 53 yrs.

In both groups osteotomy was performed using segmental sandwich technique [7].

For osteotomy in the anterior segment of the mandible, a mucosal incision was made in the depth of the vestibule from the premolar region to the premolar in opposite region. Following subperiosteal dissection, the bone region was made accessible. Anterior segmental osteotomy in relation to the mental hole was done and then osteotomy with a saw, two vertical and one horizontal incisions, as a U shape, was made (**Figure 1**). The upper mobile segment was shifted upwards approximately 8 to 10 millimeter and fixed by two miniplate then space between the mobile segment and the anterior basilar of the mandible was filled with an autogenic or allogenic graft and fixed with two miniplates. It was finally incision sutured with a 3 - 0 silk thread (**Figure 2**).

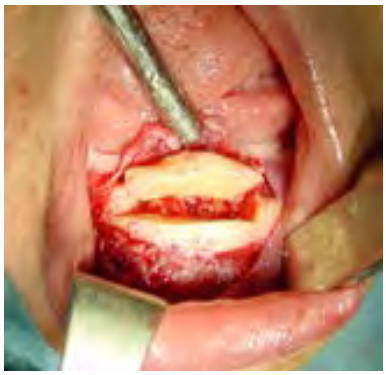

Figure 1. Osteotomy in the anterior segment of the mandible with sandwich technique.

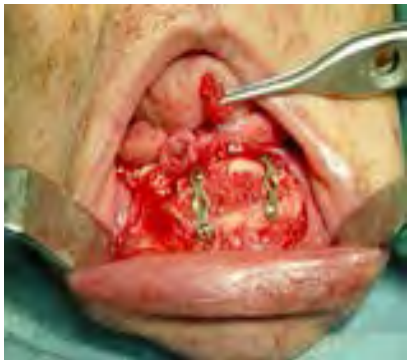

Figure 2. Anterior basilar of the mandible was filled with an autogenic or allogenic graft and fixed with two miniplates.

In group A, after the tibia bone graft was initially prepared as follows; in the metaphysic region which has cancellous bone, an oblique cut was made. After reaching the tibia region, a trephine drill with 12 mm diameter was made in the cortical bone of the cavity; some cancellous bone was harvested and skin also sutured. The valve was then fixed in its position and sutured with a 3 - 0 absorbable suture [8] (**Figure 3**).

It is worth mentioning that the level of ridge upward shift was similar in all patients, equal to the height of the plates used. During interpositional osteotomy, it was necessary that the lingual soft tissue connections to the osteotomy segment remained intact, so that the blood flow of the mobile part was not disrupted.

Panoramic radiographic views were taken from all patients before surgery (**Figure 4**), and immediately after surgery (**Figure 5**) at the 3 and 6 month follow-up visits. Three months after surgery, the patients returned with a control OPG and underwent miniplates removal surgery. During the removal of screws and plates in all 18 patients, the bone had desirable consistency in the receptor site.

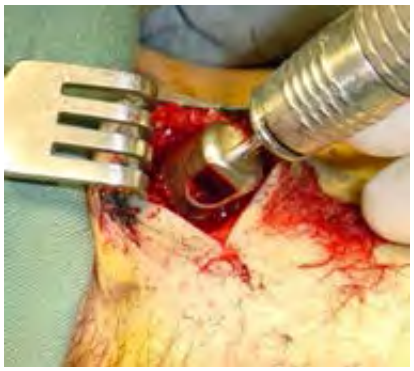

Figure 3. Tibia bone graft with trephine drill.

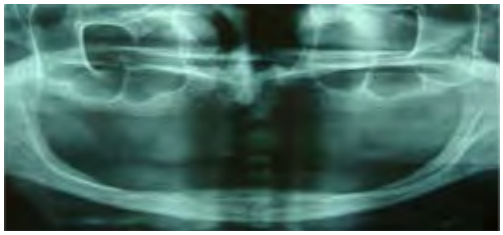

Figure 4. Initial OPG before surgery.

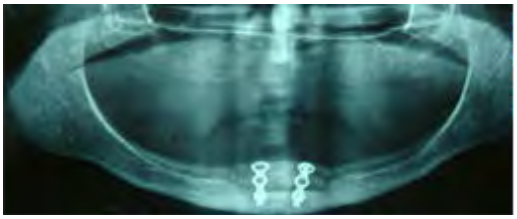

Figure 5. Immediately after surgery.

After 6 months all OPGs were studied and compared, in each OPG three points in the grafted site were selected (Figure 6). The choice of 3 points was due to having a higher confidence coefficient.

The selected M or middle point is the midline in the OPG and the R and L points are in 10 mm distance from the midline turning to the right or left. The length of these lines was measured with a caliper.

In the initial OPG of each patient, the distance between each two points was studied and compared with its related points in the 3- and 6-month follow-up OPGs.

3. Results

To compare the mean of the study data between the two allograft and autograft materials, the general liner model was used. In the middle point, changes during 6 months in both the allogenous and autogenous methods was statistically significant ($P = 0.002$). However, no significant difference was observed between the two methods regarding changes in ridge height augmentation ($P = 0.317$).

In the selected left point (L), the height of the mandibular ridge, 3 months after the allogenic bone graft had increased from 15.83 ± 1.89 to 20.33 ± 1.53 mm, showing statistical significance (Table 1). Moreover, the height of the ridge 3 months after the autogenous graft increased from 17.33 ± 0.58 to 24.33 ± 0.58 mm (Table 2), again indicating a significant difference in comparison to that before grafting. Furthermore, in the left point, regarding the changes in the ridge height during 6 months after grafting, although these changes were statistically significant in both the allogenous and autogenous grafts ($P = 0.001$), yet no meaningful difference was observed between the two methods regarding ridge height augmentation ($P = 0.128$).

In the selected right point (R) the mandibular ridge height three months after the allogenic graft increased from 15.67 ± 2.08 to 21.33 ± 1.04 mm, indicating a significant difference (Table 3). An increase from 17.17 ± 0.76 to 24.33 ± 0.58 mm was recorded in the ridge height 3 months after the autogenic graft, again being statistically significant. In the R point, during the 6 months after grafting, although the changes in both the allogenic and autogenic methods were statistically significant ($P = 0.003$), yet regarding the inferior ridge height augmentation, no significant difference was achieved between the two methods ($P = 0.332$) (Table 4).

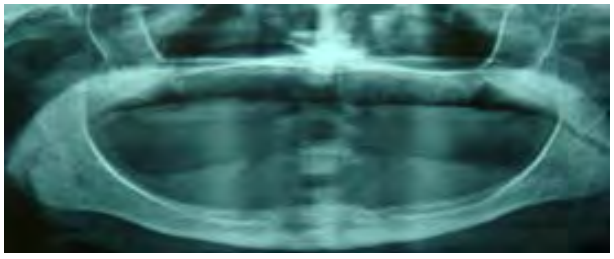

Figure 6. 6 months after surgery.

Table 1. Mean and 95% confidence interval, ridge height in the L point and its value following allogeneic grafting during, 3 and 6 months after surgery.

Average changes in height of ridge height with allograft	Time interval after surgery (month)	*P* value
15.83 ± 1.89	Immediately	$P < 0.05$
20.33 ± 1.53	3	$P < 0.05$
20.00 ± 1.00	6	$P < 0.05$

Table 2. Mean and 95% confidence interval, ridge height in the L point and its value following autogen grafting during, 3 and 6 months after surgery.

Average changes in height of ridge height with autogenic grafting	Time interval after surgery (month)	*P* value
17.33 ± 0.58	Immediately	$P < 0.05$
24.33 ± 0.58	3	$P < 0.05$
22.83 ± 1.26	6	$P < 0.05$

Table3. Mean and 95% confidence interval, mandibular ridge height in the R point and its value following allogeneic grafting during, 3 and 6 months after surgery.

Average changes in height of ridge height with allograft (mm)	Time interval after surgery (month)	*P* value
15.67 ± 2.08	Immediately	$P < 0.05$
21.33 ± 1.04	3	$P < 0.05$
20.50 ± 0.5	6	$P < 0.05$

Table 4. Mean and 95% confidence interval, mandibular ridge height in the R point and its value following autogenic bone grafting during, 3 and 6 months after surgery.

Average changes in height of ridge height with autogenic graft (mm)	Time interval after surgery (month)	*P* value
15.83 ± 1.89	Immediately	$P < 0.05$
21.33 ± 1.53	3	$P < 0.05$
20.86 ± 0.76	6	$P < 0.05$

Regarding the M point, the mandibular ridge height 3 months after the allogenic graft surgery increased from 15.83 ± 1.89 to 21.33 ± 1.53 mm, indicating a significant difference (Table 5). Moreover, the ridge height 3 months after the autogenic graft surgery increased from 18.00 ± 0.87 to 24.83 ± 1.26 mm, demonstrating a statistically significant difference (Table 6).

4. Discussion

Various indicators have been introduced in different studies regarding the success rate of bone graft surgeries. Barone *et al.* mentioned no significant localized infection at the site of transplantation, and graft attachment to the receptor's jaw, the absence of radiolucent surfaces, non-hemorrhage from the site of the

Table 5. Mean and 95% confidence interval, ridge height in the M point and its value following allogeneic grafting during, 3 and 6 months after surgery.

Average changes in height of ridge height with allograft (mm)	Time interval after surgery (month)	*P* value
15.83 ± 1.89	Immediately	$P < 0.05$
21.33 ± 1.53	3	$P < 0.05$
20.86 ± 0.76	6	$P < 0.05$

Table 6. Mean and 95% confidence interval, mandibular ridge height in the M point and its value following autogenic bone grafting during, 3 and 6 months after surgery.

Average changes in height of ridge height with autogenic graft (mm)	Time interval after surgery (month)	*P* value
18 ± 0.87	Immediately	$P < 0.05$
24.83 ± 1.26	3	$P < 0.05$
24.67 ± 1.67	6	$P < 0.05$

bone graft and the possibility of successful dental implant placement as the main indicators. They reported a success rate of 96.8% in their study. Various indicators have been introduced in different studies regarding the success rate of bone graft surgeries [9].

In our study in spite of that no statistics are presented on the possible complications and the outcomes of the two techniques, yet due to no reported cases of infection, no case of radiolucent surface and the surgeon's satisfaction of the experimental surgical outcome, it seems that the success rate was acceptable in both grafting materials. Few studies have been conducted on ridge height augmentation and the bone space resulting from the bone grafts. In the study by Chiapasco *et al.* the mean increase in the mandibular ramus in autogenic grafts was 4.6 mm; 8 to 11 mm increase in the vertical height of calvaria bone grafts was also reported. Regarding the horizontal plane [10], Roccuzzo *et al.* compared the width increase following autogenic bone grafts of the ramus alone and those of the mandible with the titanium membrane support. Their study showed a mean increase of 4.8 mm in those with a titanium membrane and 3.6 mm in the grafts without this membrane, indicating a significant difference [11].

The second finding was that during the 6-month from the grafting, remarkable healing had occurred at the graft site. It seems that considering 6 months as the graft healing time was an acceptable time in our study. Most of similar studies have reported 4 to 6 months as the required graft healing time [9] [10] [11] In the study by Nelson *et al.*, the implant was placed after a 3-month period. The biopsy provided from the recipient's bone and the histological analyses indicated a favorable graft placement for the implant [12]. Some studies have also investigated the results of immediate implant placement after grafting, yielding different outcomes. McCarty *et al.* inserted two immediate implants in the autogenic graft site in two patients; it was unsuccessful in one case [13]. However, Izuku *et*

al. achieved 100% success by placing an immediate implant in the autogenic graft site [14]. Schliephake *et al.* concluded that the graft survival rate in women is significantly lower compared to men [15]; due to the small sample size we did not consider the gender predominance in our study. In a systematic review of 13 studies on the ridge augmentation techniques for placing implants, 6 studies had investigated bone augmentation both in the vertical and horizontal planes, 4 had studied the implant placing skills and 3 others had studied grafting for the treatment of non-parallel implants. In general, no difference was observed between these techniques [16].

A systematic review by Aghaloo *et al.* in 2007 studied the techniques with the highest success rate for preserving the alveolar bone for dental implants and also the highest success rate of graft survival. The studies conducted between 1980 and 2005 were studied. In the maxila sinus, 5128 dental implants with a follow up time of 12 to 102 months were placed. The survival of the autogenic and allogenic grafts was 97% and 93.3% respectively; it was 81% in the alloplast and alloplast xenograft techniques whereas it was 96.6% in the xenograft technique alone. Regarding ridge height augmentation 2620 implants with a follow up period of 5 to 74 months were studied in which the implant survival was 95.5%, 90.4%, 94.7% and 83.8%, in the aforementioned techniques, respectively [17].

Motamedian and colleagues in a systematic review in 2016, studied the success rate of implants in the autogenic block bone in comparison to the allogenenic ones. The success rate in the autogenic bone grafts was 73.8% to 100% and 72.8% to 100%, respectively. The same figures were 93.3% to 100% and 93.7% to 100% for allogenic bones. However, due to the need for studies with a longer duration, no definite conclusion was drawn [18].

Metrens *et al.* performed a study on the short-term loss following vertical bone augmentation by calvaria in comparison to iliac bone. They concluded that calvaria bone has more consistyency in the primary repairing metal in comparison to the iliac bone [19].

Draenert and colleagues conducted a study on the vertical bone augmentation by GBR, local autogenic block, pizosurgery modification techniques and pelvis bone block in which the outcome was the wide popularity of the GBR technique and autogenic bone block with minimum cortex thickness and a large volume of particulated material [20].

In a clinical and histopathological study by Rocchietta *et al.* regarding vertical bone augmentation with the autogenous block or autogenic particles in combination with GBR, both the mentioned methods following implant and prothesis placement showed a high success rate. However, histopathological study showed a better connection of autogenic bone in relation to allografts [21].

5. Conclusions

The present study can be verified from several aspects. Although the two grafting material demonstrated no difference regarding the changes in the ridge

height, they were very effective in providing the suitable space in the mandible with the aim of removing atrophy or implant placement.

Regarding our findings it can be concluded that both the autogenic and allogenic grafting materials are efficient in ridge height augmentation with the aim of mandibular atrophy rehabilitation or implant placement. No significant difference in terms of the success rate and the recovery time was observed between the two methods.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Meta-Analysis of Clinical Outcomes of Lumbar Fusion Surgical Interventions for Degenerative Spondylolisthesis

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Abstract

Introduction: Surgical interventions for degenerative spondylolisthesis are varied with comparable claims of success. Fusion based technique is one of the most commonly used surgical interventions in treating this condition. The aim of this meta-analysis is to compare the effectiveness of the Lumbar Interbody Fusion techniques (specifically Posterolateral Interbody approach—PLIF) versus Posterolateral Instrumented Fusion (PLF). The clinical outcomes investigated were: back pain, leg pain, function, Oswestery Disability Index (ODI), Disability Rating Index (DRI), fusion and revision rates if reported. **Methods:** Combinations of keywords and MeSH terms, where appropriate, were used to search for studies in Medline via Ovid, Embase, Cochrane Library, and Google scholar. The initial search was conducted on 10 August 2016 and updated on 13 June 2017. Eligibility criteria for the studies to be selected for this meta-analysis were: Randomised Controlled Trials (RCTs), cohort and consecutive cases studies that compared at PLIF versus PLF surgical interventions at the lumbar region. Heterogeneity indicators and Forest plot were computed using RevMan 5. **Results:** Out of the initial hits of 3021, 5 articles were selected as relevant and assessed for risk of bias and then data was extracted and tabulated. These 5 studies reported data from (900 patients' records, follow up ranges from 6 months to 5 years) undergone one of 2 interventions (PLIF or PLF). The overall effect for ODI and leg pain showed no advantage of any intervention over the other while there was a greater odd ratio of fusion if the operation applied PLIF techniques (Overall Z = 2.86, p = 0.004). **Conclusions:** There is a need for more high quality clinical trials to compare these two interventions. However, available data indicate that there

are comparable results in the main clinical outcomes between PLIF and PLF. PLIF has superior fusion rate which does not seem to affect post-operative pain ratings.

Keywords

PLIF, PLF, Lumbar Spine, Pain, Spondylolisthesis, Fusion Techniques, Review

1. Introduction

Spinal disorders at the lumbar region affect around 11% - 15% of the population worldwide [1]. Causes of disc problems at the lumbar region include a degenerative process because of ageing, spinal deformities and instability such as spondylolisthesis. Spondylolisthesis is usually defined as a forward displacement of one of the lower lumbar vertebrae over the vertebra beneath it. When this occurred the resulting pressure on the spinal nerves could lead to pain and other symptoms including a loss of mobility and loss of sensory and motor functions along the nerve pathways [1]. Surgical interventions for degenerative spondylolisthesis are varied with comparable claims of success. Fusion based technique is one of the most commonly used surgical interventions in treating this condition. There are two major approaches for the fusion techniques namely Interbody fusion (specifically Posterolateral Interbody approach—PLIF) and Posterolateral Instrumented Fusion (PLF). In interbody fusions there are three common techniques; Posterior lumbar, Transforaminalor Anterior. However, clinical outcomes of these interventions are inconsistently reported in trials that compare the effectiveness of these interventions. The aim of this meta-analysis is to compare the effectiveness of the Lumbar Interbody Fusion techniques versus Posterolateral Instrumented Fusion (PLF). The clinical outcomes investigated are: back pain, leg pain, function, Oswestery Disability Index (ODI), Disability Rating Index (DRI), fusion and revision rates if reported.

2. Methods

Combinations of keywords and MeSH terms, where appropriate, were used to search for studies in Medline via Ovid, Embase, Cochrane Library, and Google scholar. The lists of keywords were (lumbar disc/disk or disc/disk disease, discectomy, posterior, posterolateral, transforaminal, anterior, spondylosis, spondylolisthesis) and (vertebr*, lumbar spine, spine) and (pain, disability, quality of life, outcome). The initial search (No time or language limits) was conducted on 10 August 2016 and updated on 13 June 2017. Eligibility criteria for the studies to be selected for this meta-analysis were: Randomised Controlled Trials (RCTs), cohort and consecutive cases studies that compared at PLIF versus PLF surgical interventions at the lumbar region. Initial screening of titles was conducted by one reviewer (SG) and screening of full articles and data extraction

was conducted by two reviewers independently with a third reviewer acting as an arbiter in case a disagreement between the two systematic reviewers on the inclusion of a study arises. The decision process to select the relevant studies was as follows:

1) Title and abstracts from databases were managed by Endnote library.

2) If the study is not an RCT, case-control or cohort study involving human patients then it is excluded.

3) If the study does not include a surgical intervention, then it is excluded.

4) If the surgical technique is not a fusion technique, then it is excluded.

Data were extracted by two systematic reviewers and checked by other contributors. The data which extracted from the selected studies included; the study design, grades of the disc disease and the spinal levels affected, number of patients and their age, length of the follow up period after surgery. Data on the fusion rate at the spinal level, outcome score (SF12, SF36), ODI, revision rate, patients' satisfaction and both radiating and back pain on a visual analogue score are also extracted and tabulated.

The risk of bias in the selected articles was assessed using the Cochrane collaborations guidelines. Two reviewers assessed the articles independently and then convened to produce the risk of bias judgment using RevMan 5 software [2]. Any disagreements between the two reviewers were resolved by asking a third reviewer to provide further judgment on the article in question. Heterogeneity indicators were calculated using RevMan 5 and it has been decided if pooling of data on any clinical outcome was required that a fixed effect model should be applied if the heterogeneity indicator I^2 was less than 70% and at least three studies provided suitable data for meta-analysis [3] [4]. Publication bias was assessed visually after producing Funnel plots.

3. Results

Out of the initial hits of 3021 hits 46 articles were deemed relevant (**Figure 1**). Forty-one full length articles were excluded with reasons such as; being a discussion article or report on experiments on cadavers or examination of learning curve of surgeons. Five articles were assessed for risk of bias (**Table 1**) and then data was extracted and tabulated. These 5 studies reported data from (900 patients' records, follow up ranges from 6 months to 5 years) undergone one of the 2 interventions (see **Table 2** for full characteristics of the studies). Patients were matched for sex and age. All studies lacked description of randomisation process and quality of articles ranged from intermediate to poor. Five studies have not reported pain outcomes or Oswestry Disability Index (ODI) scores. Those reported ODI or pain outcomes found no significant differences between the compared interventions. There were no significant differences.

3.1. Studies' Conclusion

Authors of 3 out of the five studies included agreed that overall PLIF resulted in

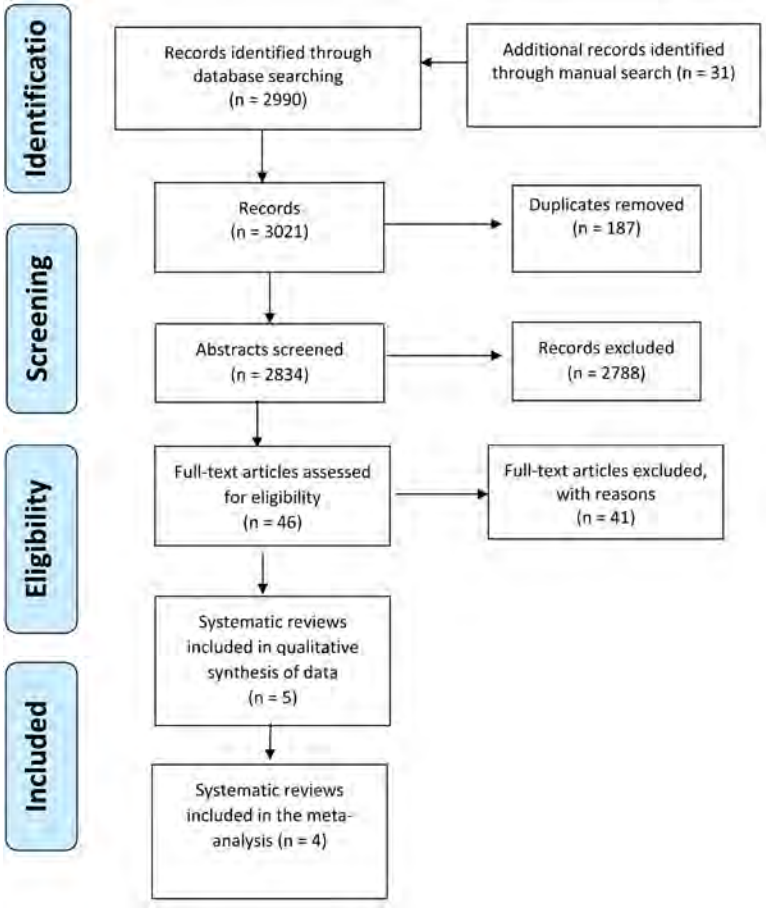

Figure 1. PRISMA flow chart showing results of the selection process.

Table 1. Risk of bias summary: review authors' judgements about each risk of bias item for included studies. Red circle = high risk of bias, Green circle = low risk of bias, Yellow circle = unclear risk of bias.

Study	1	2	3	4	5	6	7
Dehoux *et al.*	-	-	-	-	-	-	-
Ekman *et al.*	-	-	-	-	-	-	-
Farrokhi *et al.*	-	-	-	-	-	-	-
Lee *et al.*	-	-	-	-	-	-	-
Musluman *et al.*	-	-	-	-	-	-	-

1—Random sequence generation (selection bias). 2—Allocation concealment (selection bias). 3—Blinding of participants and personnel (performance bias). 4—Blinding of outcome assessment (detection bias). 5—Incomplete outcome data (attrition bias). 6—Selective reporting (reporting bias). 7—Other bias.

similar clinical results as PLF [5] [6] [7] but better fusion rate and better maintenance of reduction is more likely to be obtained post PLIF [5] despite the fact that PLIF could lead to more complications [6]. Farrokhi *et al.* 2012

Table 2. Summary of characteristics of studies included and the reported clinical outcomes.

Study	Dehoux *et al.* 2004	Ekman *et al.* 2007	Farrokhi *et al.* 2012	Lee *et al.* 2014	Musluman *et al.* 2011
Study design	NRCT	NRCT	RCT	Prospective Randomized study	RCT
Grades/levels included	1 - 3/N/A	1 - 3/L3-L5	N/A/L3-S1	1 - 2/L4-S1	1 - 2/L3-S1
Number of Patients PLIF/PLF	27/25	86 vs 77	40 vs 40	42 vs 39	25 vs 25
Age (years) PLIF/PLF	39.5 vs 42.4	40 vs 39	50.4 vs 49.7	53.4 vs 53.7	50.6 vs 47.3
Follow up period	6 years	2 years	1 year	>2 years	Up to 6 years.
Fusion rate (%) PLIF/PLF	93 vs 68	N/A	89.1 vs 66.7	90.4 vs 89.7	96 vs 80
ODI PLIF/PLF	N/A	25 vs 25	17 ± 12.98 post surgery (PLIF) 25.34 ± 9.36 (PLF)	38.9 ± 9.1→9.0 ± 1.6 vs 37.5 ± 9.4→8.6 ± 1.3	30.20 ± 5.70 →13.60 ± 1.95 vs 29.20 ± 6.42 → 18.20 ± 3.65
DRI PLIF/PLF	N/A	47→30 vs 49→29	N/A	NA	N/A
Pain VAS PLIF/PLF	N/A	35 vs 37	1.2 ± 1.58 vs 1 ± 0.98	8.7 ± 1.3→1.5 ± 1.2 vs 8.5 ± 1.4→1.6 ± 1.0 (Lower Back Pain) 6.4 ± 2.1→0.9 ± 0.3 vs 5.9 ± 2.3→1.0 ± 0.4 (Radiating Pain)	1.00 ± 0.64 vs 1.08 ± 0.90 (Leg Pain) 1.20 ± 0.57 vs 1.8 ± 0.57 (Back Pain)
Patients satisfaction PLIF/PLF	77% vs 68%	PLF > PLIF	PLF > PLIF	N/A	N/A
Clinical and Mechanical outcomes	Clinically PLIF = PLF Mechanically PLIF better than PLF	PLIF = PLF mechanically But with more complications	PLF is better than PLIF clinically	PLIF = PLF	PLIF better mechanically PLF is better clinically
Overall conclusion of authors					

NRCT = non-randomized controlled trial, ODI = oswestery disability index, VAS = visual analogue scale, DRI = disability rating index.

concluded that PLF provides better clinical outcomes and more improvement in the lower back pain compared to PLIF despite the low fusion rate of PLF [8]. Musluman *et al.* 2011 suggested that PLIF provided more solid mechanical construct compared to PLF [9]. But PLF exhibited better clinical outcomes at an earlier stage, including improvements in quality of life pain relief and functional ability.

There is also an agreement between all the studies that additional studies with a larger sample size should be performed to better understand the clinical and

radiological outcomes of both techniques.

3.2. Meta-Analysis Findings

It was very difficult to judge if there was publication bias because of the low number of studies in different outcomes. It was possible to pool data in three outcomes Oswestery Disability Index (ODI) (Figure 2), leg pain (Figure 3) and fusion rate (Figure 4). See Appendix 1 for full calculations and data. The overall

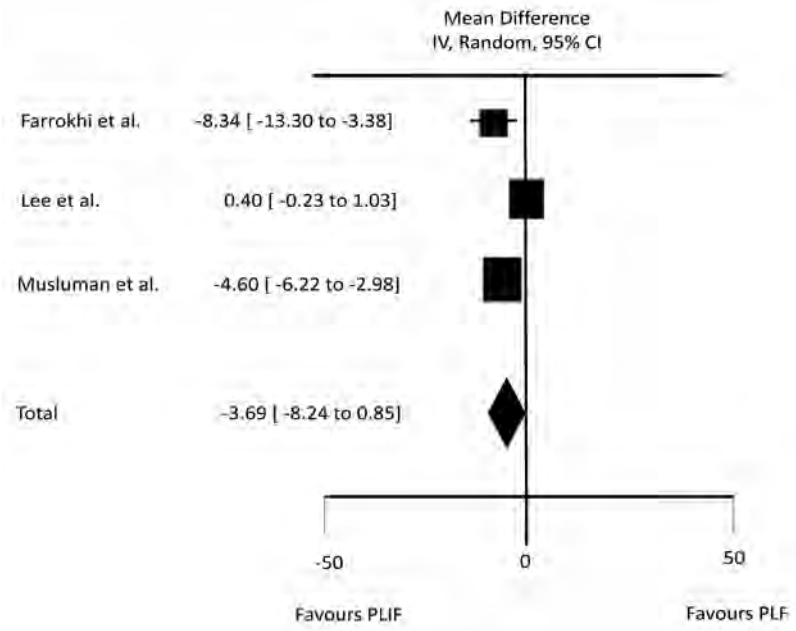

Figure 2. Forest plot of comparison of ODI post-surgery.

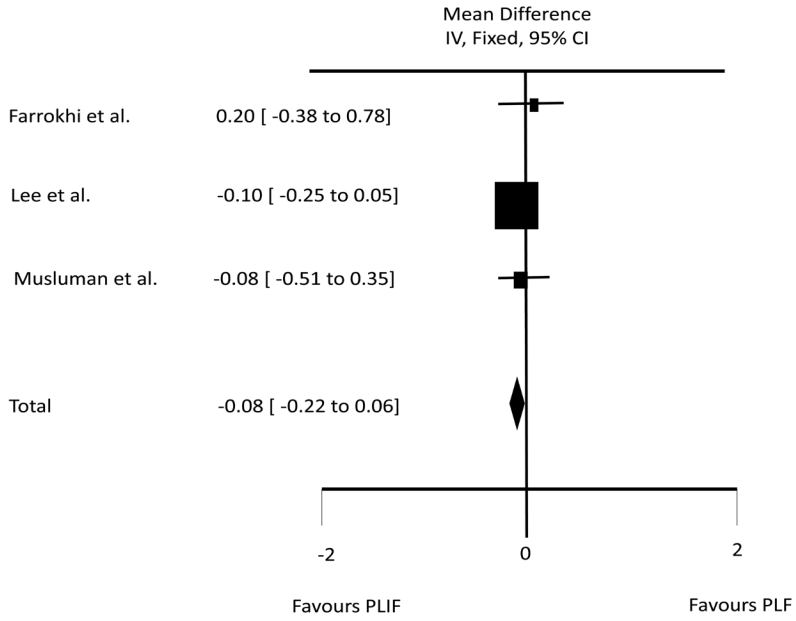

Figure 3. Forest plot of comparison between visual analogue scale (0 to 10) post operations of leg pain.

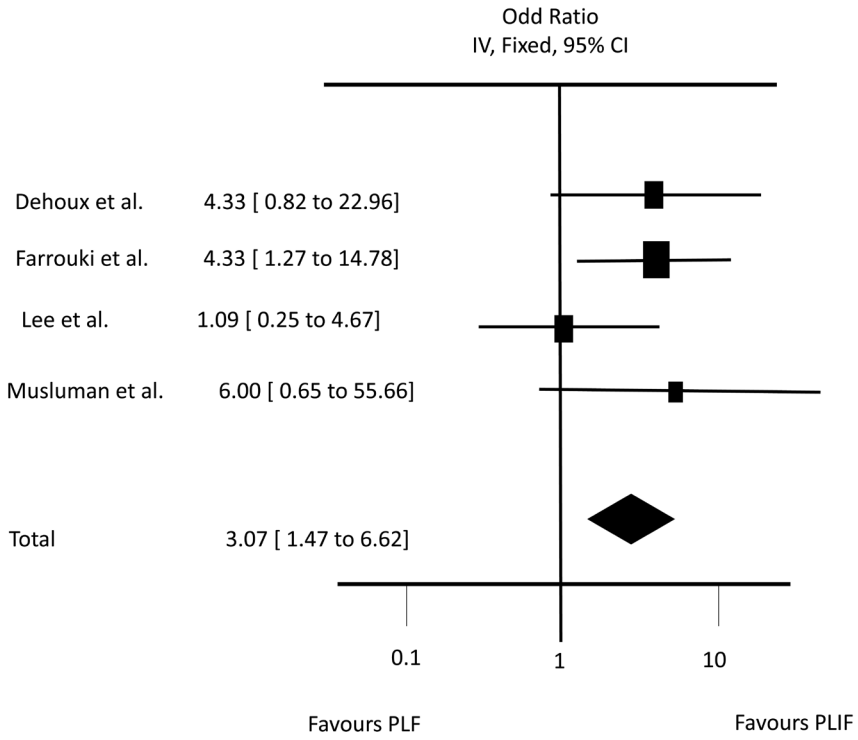

Figure 4. Forest plot of comparison between the odd ratio of fusion rate outcome in the two interventions. There is a greater odd ratio of fusion if the PLIF technique was applied (Overall Z = 2.86, $p = 0.004$).

effect for ODI and leg pain showed no advantage of any intervention over the other while there was a greater odd ratio of fusion if the operation applied PLIF techniques (Overall Z = 2.86, $p = 0.004$).

3.3. Complications

Overall there were slightly more complications post PLIF operations. There is some evidence that PLIF procedure resulted in more bleeding and is more invasive [6] and the leak of cerebrospinal fluid was 14% more in post PLIF than post PLF with a slightly greater risk of infection and slightly greater probability of permanent motor impairment in post PLIF patients [8]. Blood Loss (ml) 360 ± 30 vs 350 ± 25, Operation time (Hours) 2.6 ± 0.3 vs 2.1 ± 0.2, Hospital stay (Days) 4.7 ± 2.2 vs 4.8 ± 1.7, incision length (cm) 8.6 ± 1.7 vs 8.1 ± 1.5.

4. Discussion

Findings from the 5 articles selected and analysed in this review suggested that fusion rate is slightly better post PLIF despite the likelihood of higher complications with this intervention. In the four studies that reported fusion rates, three studies reported a fusion rate post PLIF to be at least 16% more efficient than post PLF. This finding is in agreement with the meta-analysis outcome of Ye *et al.* 2013, who found that PLF generally produced less fusion rates than PLIF [10]. Clinically the fusion rate post lumbar surgery for spondylolisthesis is the main

goal of the intervention as it usually linked to the favourite clinical outcomes [11]. However, as far as other clinical outcomes are concerned the two interventions are comparable to each other. While the selected studies did not discussed reasons behind a choice of one intervention over the other it is clear that surgeon preference and experience and anatomical consideration determine what type of surgery is performed.

Despite the significant difference between PLIF and PLF in the fusion rate the pain outcomes, whether it was leg or back pain, was similar after the surgery in the two groups of patients. This was also the findings of [10] and [12]. This is unexpected in the case of back pain as a better fusion rate should have an impact on the persistence of this type of pain. Differences between the follow-up in the selected studies only partially could explain why there was no differences between PLIF and PLF in visual analogue scale of back pain after surgery. However, the similarity of the pain ratings between the two interventions can be explained by the fact that the two surgeries achieve satisfactory spinal nerve root decompression. Our findings should be cautiously interpreted because the studies selected for this meta-analysis lacked description of randomisation process and not all outcomes were reported. While it is accepted that blinding surgeons is impossible the studies failed to blind patients. Other limitation of this meta-analysis is the differences between studies in the follow-up period.

5. Conclusion

There is a need for more high quality clinical trials to compare these two interventions. However, available data indicate that there are comparable results in the main clinical outcomes with PLIF providing superior fusion rate which does not seem to affect post-operative pain ratings.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Appendix 1: Data Used to Produce Effect Size and Forest Plot

Table (a). Data used to produce Forest plot output in RevMan 5 to compare ODI post-surgery.

	PLIF			PLF			
Study	mean	SD	N	Mean	SD	N	Weight
Farrokhi *et al.*	17.0	12.98	40	25.34	9.36	40	26.1%
Lee *et al.*	9.0	1.6	42	8.6	1.3	39	37.7%
Musluman *et al.*	13.6	1.96	25	18.2	3.65	25	36.2%
Total (95% CI)			107			104	100.0%
Heterogeneity: $Tau^2 = 14.15$; $Chi^2 = 41.37$, df = 2 ($p < 0.00001$); $I^2 = 95\%$							
Test for overall effect: Z = 1.59 ($p = 0.11$)							

Table (b). Data used to produce Forest plot output in RevMan 5 to compare between visual analogue scale (0 to 10) post operations of leg pain.

	PLIF			PLF			
Study	mean	SD	N	Mean	SD	N	Weight
Farrokhi *et al.*	1.2	1.58	40	1	0.98	40	6%
Lee *et al.*	0.9	0.3	42	1	0.4	39	83.3%
Musluman *et al.*	1	0.64	25	1.08	0.9	25	10.7%
Total (95% CI)			107			104	100.0%
Heterogeneity: $Chi^2 = 0.97$, df = 2 ($p = 0.62$); $I^2 = 0\%$							
Test for overall effect: Z = 1.11 ($p = 0.27$)							

Table (c). Data used to produce Forest plot output in RevMan 5 to compare log odd ratio of Fusion Rate outcome. There is a greater odd ratio of fusion if the PLIF techniques were applied. (Overall Z = 2.86, $p = 0.004$).

	Log OR	SE	Weight
Dehoux *et al.*	1.5	0.85	21.2%
Farrokhi *et al.*	1.5	0.63	39.2%
Lee *et al.*	0.08	0.74	27.7%
Musluman *et al.*	1.8	1.14	11.9%
Heterogeneity: $Chi^2 = 2.76$, df = 2 ($p = 0.43$); $I^2 = 0\%$			
Test for overall effect: Z = 2.86 ($p = 0.004$)			

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Reduction of Pain, Fatigue, Gastrointestinal and Other Symptoms and Improvement in Quality of Life Indicators in Fibromyalgia Patients with Membrane Lipid Replacement Glycerolphospholipids and Controlled-Release Caffeine

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Abstract

Objectives: A preliminary, open label study was initiated to determine if oral wafers containing a combination of membrane glycerolphospholipids and controlled-release caffeine could reduce self-reported pain, fatigue, and gastrointestinal symptoms and improve quality of life (QOL) indicators in fibromyalgia patients. **Methods:** Pain, fatigue and other symptoms were determined using validated, patient survey forms completed over an 8-day test period and compared to baseline values. Participants included 21 patients (15 females and 6 males) of average age of 48.5 ± 9.8 years with a diagnosis of fibromyalgia. These patients consumed four daily chewable wafers containing glycerolphospholipids (4.8 g) and one controlled-released caffeine (184 mg) wafer that maintained caffeine levels at approximately one cup of coffee for over 8 h. **Results:** Participants in the study responded to the combination test supplement within days. By the end of the study there were significant overall improvements (36.1%, $p < 0.001$), reductions in pain (27.2%, $p < 0.001$), fatigue (37.8%, $p < 0.001$), gastrointestinal symptoms (54.7%, $p < 0.001$) and improved ability to complete tasks and participate in activities (quality of life indicators) (39.1%, $p < 0.001$). Regression analysis of the data using a generalized mixed-effects model and calculating R^2 values indicated that reductions

in pain, fatigue and gastrointestinal symptoms and improvements in quality of life indicators were consistent, and occurred with a low degree of variance. Males responded slightly better to the combination supplement than females but for most parameters these differences were not significant. **Conclusions:** The combination membrane lipid replacement glycerolphospholipid supplement with controlled-release caffeine was safe and effective and significantly reduced pain, fatigue and gastrointestinal symptoms as well as improved QOL indicators in fibromyalgia patients.

Keywords

Pain, Fatigue, Gastrointestinal Symptoms, Quality of Life, Phospholipids, Caffeine

1. Introduction

Fibromyalgia is characterized by chronic, widespread pain, abnormal processing of pain and increased sensitivity to external stimuli, along with fatigue, gastrointestinal symptoms and changes in memory, mood and sleep [1] [2] [3]. In 2010 the American College of Rheumatology established diagnostic criteria for the diagnosis of fibromyalgia based on a pain index and symptom severity scale [2]. Using this diagnostic criteria it has been estimated that between 0.1% - 3.3% of the populations in western countries and 2.0% of the population of the United States have fibromyalgia, with higher incidence rates in females compared to males [4].

In the last few years natural supplements have been used to reduce symptoms in patients with fibromyalgia, chronic fatigue and other chronic illnesses [5]; however, few if any of these natural supplements were considered effective [6]. Some symptoms, such as fatigue and pain, also occur naturally during aging, and they are important secondary conditions in many chronic diseases [7].

Pain and fatigue are related functionally to cellular energy systems found primarily in mitochondria and specifically in the electron transport chain of the mitochondrial inner membrane [8] [9]. Damage to mitochondrial membranes occurs in various diseases, mainly by oxidation of phospholipid components, and this can result in ion leakage across inner mitochondrial membranes and reductions in the ability of mitochondria to produce high-energy molecules [10] [11]. During aging and most chronic diseases the production of oxidative free radicals, such as Reactive Oxygen and Nitrogen species (ROS/RNS) and other molecules, can cause oxidative stress and cellular damage [10] [11] [12]. ROS/RNS damage often occurs to cellular membranes, and in particular, to the glycerolphospholipids of mitochondrial membranes [11] [12] [13] [14].

Membrane Lipid Replacement (MLR) therapy plus antioxidants has been used to replace damaged glycerolphospholipids in various clinical disorders and in aged individuals [8] [14] [15]. MLR results in the replacement of damaged cellu-

lar lipids with undamaged, unoxidized lipids to ensure proper function of cellular and organelle membranes. Combined with antioxidants, MLR supplements have proven to be effective in reducing ROS/RNS-associated changes in cellular activities and functions and for reducing symptom severity and providing host support in various clinical conditions [8] [14] [15] [16]. In fibromyalgia patients, the MLR supplement NTFactor Lipids® has been used to reduce fatigue [8] [16]. In a preliminary case report pain, fatigue and gastrointestinal symptoms were reduced in a small number of fibromyalgia patients (P.A. Breeding and G.L. Nicolson, in preparation)

In fibromyalgia patients another natural approach to reducing the severity of symptoms has been to use low-dose caffeine (equivalent to one or less than one cup of coffee or about 40 - 50 mg of caffeine) to moderate doses (equivalent to 1.5 - 2.5 cups of coffee) of caffeine to reduce pain and fatigue [17]. In the study of Scott *et al.* [17] caffeine consumption had a modest but significant effect on chronic pain reduction compared to an absence of caffeine consumption, but among non-opioid users this effect was not significant [17]. Caffeine is one of the most widely consumed natural food supplements in the world, and it is present in numerous foods and beverages. Caffeine is generally considered safe at doses up to 400 mg per day for adults [18] [19].

Here we tested the results of a combination natural supplement that contained a formulation of MLR glycophospholipids (NTFactor Lipids®) plus a low dose of controlled-released caffeine in chewable wafers in an open-label study format that used self-reported results on pain, fatigue, gastrointestinal symptoms and quality of life (QOL) indicators in a small group of fibromyalgia patinets.

2. Materials and Methods

2.1. Materials and Methods

An open label, Institutional Review Board approved, clinical trial was initiated to study the effects of an all-natural glycerolphospholipid chewable wafer supplement (Patented Energy™ with NTFactor Lipids®) and a chewable wafer containing NTFactor Lipids® and 184 mg controlled-release caffeine (Brite-Alert™) on fibromyalgia signs and symptoms. The supplement products provided a total of 4.8 g per day of NTFactor Lipids® and 184 mg controlled-release caffeine per day (released over 8 - 9 h). The supplement products were provided by Nutritional Therapeutics, Inc. (Hauppuage, NY). The dose of caffeine in the Brite-Alert™ wafer maintains the caffeine blood level equivalent to approximately one cup of coffee over an 8 - 9 h period. NTFactor Lipids® is a patented, proprietary lipid complex containing an exogenous source of polyunsaturated phosphatidylcholine, phosphatidyglycerol, phosphatidylserine, phosphatidylinositol, and other membrane phospholipids [8]. The participants took the daily dose for 8 days: one Brite-Alert™ wafer plus one Patented Energy™ wafer in the morning and two Patented Energy™ wafers in the afternoon.

To monitor patients we constructed a Combined Fibromyalgia Symptom

Survey Form (**Supplementary Figure 1**) that was based on established, validated, published symptom survey forms for pain, fatigue, gastrointestinal symptoms and QOL indicators [20] [21] [22] [23] [24]. Each question in the survey form was answered numerically based on a linear scale from 0 to 10. One question (Q38, based on time of day when subject is most symptomatic) was deleted from the analysis due to its non-numerical response. So that the general scale remained the same in the overall survey form, from lowest (0) to highest (10) severity of symptoms, the QOL portion of the survey form used this same format, which causes improvements in QOL to be shown by lower, not higher, scores.

2.2. Subjects

Participants (male and female) were recruited online using fibromyalgia support sites on social media (Facebook). The minimum sample size necessary to determine a significant difference ($p < 0.05$) in the results was estimated to be 20 subjects. Thus 30 patients with a confirmed diagnosis of fibromyalgia [1] [2] were recruited and sent an Informed Consent document and a Protocol document that explained the trial and the requirements of subjects in the trial. Qualifying participants who signed the Informed Consent document and agreed to the trial protocol were sent an eight day supply of the test supplements and the necessary copies of the Combined Fibromyalgia Symptom Survey Form and a Protocol Form.

2.3. Study Design

Male and Female subjects of age 18 - 65 years with a confirmed diagnosis of fibromyalgia [2] and who signed an Informed Consent document and agreed to participate in the study were sent the test supplements. Each participant was instructed to take the test supplements in the morning and afternoon and complete the symptom survey form early in the evening on day 0 (the evening before starting the test supplements), day 1 (evening on the first day after taking the supplements), day 2 (evening on the second day after taking the supplements), day 4 (evening on the fourth day), day 6 (evening on the sixth day) and day 8 (evening on the eighth day). After the 8-day trial, the completed survey forms were returned by mail in a self-addressed mailer to the Lead Investigator. Participants were also advised not to change any of their daily medications, diet or routine during the study.

2.4. Statistics

Data were analyzed by analysis of variance (ANOVA), with significance defined as $p < 0.05$. Further data analysis was performed with regression analysis, with significance defined as $p < 0.05$. This was established using a generalized mixed-effects model and calculating R^2 as Marginal R^2 and Conditional R^2 values [25]. All of the statistical analyses were done indpendently by the Statistical Unit at Cornell University.

3. Results

3.1. Subjects in the Study

Of the 30 participants recruited to this open-label pilot study, 21 subjects (15 females and 6 males) were fully compliant and completed the study. The main reason for subjects not completing the study was non-compliance with trial instructions. Either subjects did not sign the Informed Consent document, or they did not take all of the study supplements, or they failed to send back all of the fully completed symptom survey forms. Most of the subjects that withdrew from the study did so without taking the test supplements. However, one participant left the trial because of severe headaches, which had occurred intermittently before the trial; one participant left because of symptoms unrelated to the trial, and one left due to cardiovascular complaints that had also occurred before the trial. In all of these cases the subjects reported that their symptoms that caused them to leave the study had occurred intermittently before starting the study.

The mean age of participants completing the study was 48.5 ± 9.8 years (16 females, 48.7 ± 10.2 years and 6 males, 48.0 ± 5.6 years, respectively). There was no significant difference in mean age between males and females or between participants completing the study and those that did not complete the study.

3.2. Effects of MLR Supplements on Fibromyalgia Symptoms and QOL

We examined the daily effects of the test supplements on self-reported symptom severity and QOL scores during the 8-day trial and found significant improvements in the individual scores on specific questions (**Supplementary Table 1**). For example, the overall mean scores (**Figure 1(A)**) for the 46 questions evaluated in the trial significantly improved during the trial (significantly lower total scores). In the case of QOL, the scale was inverted to show improvements by lower scores (**Supplementary Figure 1**). Patients positively responded to the test supplements, as shown by significant improvements in total overall scores over the 8-day study period (**Figure 1(A)**, 36.1%, $p < 0.001$).

In addition, when the scores in the subparts of the combined symptom survey form were examined for reductions in pain (**Figure 1(B)**, 27.2%, $p < 0.001$), fatigue (**Figure 1(C)**, 37.8%, $p < 0.001$), and gastrointestinal symptoms (**Figure 1(D)**, 54.7%, $p < 0.001$) as well as improvements in QOL indicators (**Figure 1(E)**, 39.1%, $p < 0.001$), by day 8 there were significant differences (ANOVA) from the baseline values obtained before starting the MLR supplements. These significant differences were also obtained on each day monitored during the trial compared to baseline values on all symptoms and indicators.

3.3. Analysis of Data Based on Gender

We examined the trial data to see if there were any differences between the responses to the test supplements between females and males. Both females and males responded to the test supplements, but there were some differences

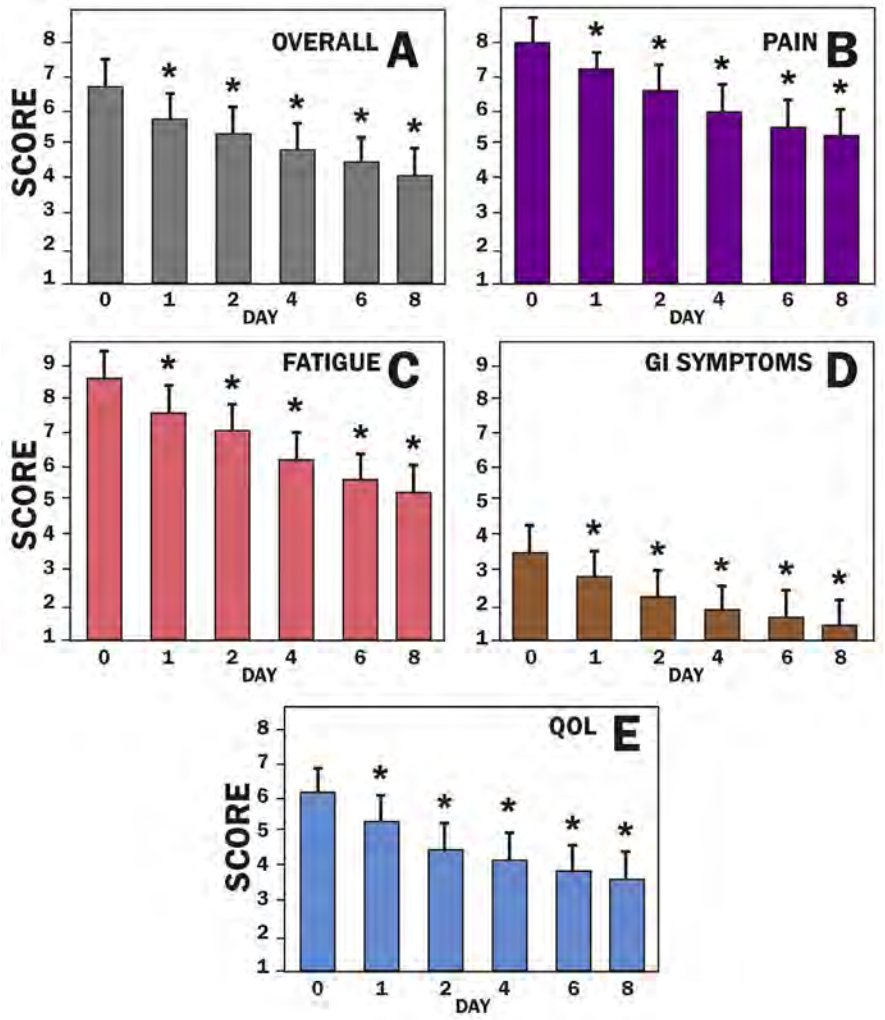

Figure 1. Combined and sub-parts of the symptom survey form scores by day for the study. Bars indicate mean ± SEM for overall scores (A), and for pain (B) fatigue (C), gastrointestinal symptoms (D), and QOL indicators (E). Note that the QOL indicator scores are shown in reciprocal format with improvements showing lower scores.

between males and females in responses overall (**Figure 2(A)**). When we examined the subparts of the symptom survey, we found some differences in responses between males and females with respect to pain (**Figure 2(B)**), fatigue (**Figure 2(C)**), gastrointestinal symptoms (**Figure 2(D)**) and QOL indicators (**Figure 2(E)**). Similar to the combined scores, females and males showed significant differences between test and baseline scores in all subcategories examined ($p < 0.001$); however the differences found between the scores of females and males did not reach significance in most analyses.

3.4. Model Analysis of the Data

We used the procedures of Nakagawa and Schielzeth [25] to calculate R^2 values for a generalized mixed-effects model. Calculations for fixed effects (gender) (Marginal R^2 values) or random effects (subjects) (Conditional R^2 values) for the

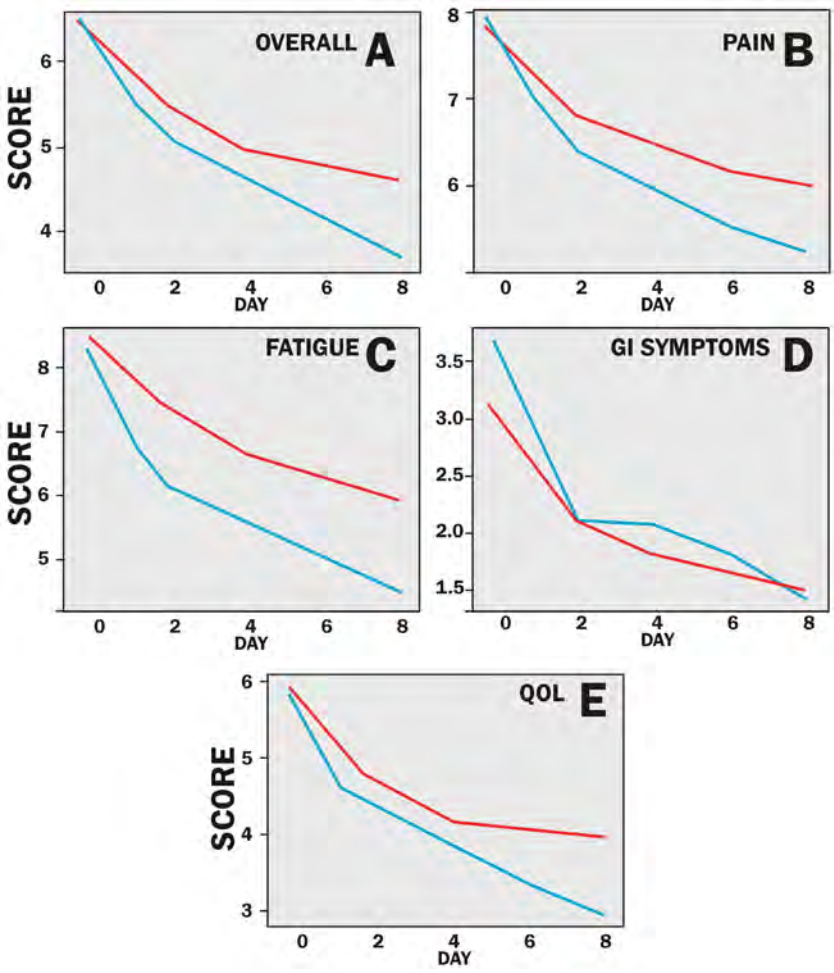

Figure 2. Collective symptom survey form scores by day and gender for the study. Plots indicate overall scores (A), and for pain (B) fatigue (C), gastrointestinal symptoms (D), and QOL indicators (E). Plots are shown for males (blue) and females (red). Note that the QOL indicator scores (E) are shown in reciprocal format with improvements showing lower scores.

various parameters yielded R^2 values for the model with day as the variable, including the baseline values at day 0. The various R^2 values were: Marginal R^2 = 0.2846, Conditional R^2 = 0.94996 (overall score); Marginal R^2 = 0.3923, Conditional R^2 = 0.9111 (fatigue score); Marginal R^2 = 0.2494, Conditional R^2 = 0.9266 (pain score); Marginal R^2 = 0.1023, Conditional R^2 = 0.9340 (gastrointestinal symptom score); Marginal R^2 = 0.1099, Conditional R^2 = 0.9691 (quality of life score). The Marginal and Conditional R^2 values indicate a low degree of variance and good consistency for the trial, even though the number of subjects in the study was limited. The Conditional R^2 scores in the generalized, mixed-effects model suggested that increasing the number of participants (subjects) in the study would be unlikely to change the results.

4. Discussion

Oral supplements that contain membrane glycerolphospholipids have been used

successfully in several clinical MLR studies [8] [14] [15] [16] [26] [27]. Here we used the MLR supplement NTFactor Lipids® with fructooligosaccahrides and antioxidants to protect the phospholipids from disruption, degradation and oxidation in the gut [14] [15]. These membrane glycerolphospholipids can be absorbed and transported into tissues and cells without excessive oxidative damage [8] [14] [15]. Once inside cells, the undamaged, replacement membrane phospholipids can exchange with damaged membrane phospholipids, resulting in removal of the latter molecules from cells. They also provide important lipid precursors for specific molecules, such as cardiolipin in the inner mitochondrial membrane. An important addition to the supplements we used was the incorporation of fructooligosaccharides and antioxidants in the MLR glycerolphospholipid mixture to avert oxidation and other types of damage to the phospholipids. This is important for maintaining potency during storage before they can be ingested and while they are ingested and dispersed into small lipid globules that are absorbed by the gut epithelium and transferred to the lymph and blood circulations for transport to various tissues and cells [8] [14] [15].

In animal studies a similar MLR supplement was used to prevent age-associated hearing loss [28]. Hearing loss is normally associated with aging, but addition of MLR glycerolphospholipids to animal chow shifted the threshold hearing from 35 - 40 dB in control aged laboratory rats to 13 - 17 dB in the treatment group ($p < 0.005$). Seidman *et al.* also found that cochlear mitochondrial function was preserved, and aging-related mitochondrial DNA deletions found in the cochlear were significantly reduced in the animals receiving the glycerolphospholipid supplement in their chow [28].

Most clinical studies using oral MLR supplements have been designed to reduce fatigue and protect cellular and mitochondrial membranes from oxidative damage [8] [14] [15] [16]. In some studies NTFactor® has been used in a vitamin and mineral mixture (Propax™) for cancer patients to reduce the adverse effects of cancer therapy, such as chemotherapy-induced fatigue, nausea, vomiting and other side effects [29] [30]. In a double-blind, placebo-controlled, randomized cross-over trial on cancer patients receiving chemotherapy MLR improved fatigue scores, nausea, diarrhea, impaired taste, constipation, insomnia and improved QOL indicators [29].

In several clinical studies MLR glycerolphospholipids have been shown to significantly reduce fatigue [8] [14] [15] [16] [27] [31] [32] [33]. For example, using aged subjects with chronic fatigue in a cross-over format, fatigue scores were significantly reduced from severe to moderate after eight weeks of MLR supplementation with NTFactor®. Mitochondrial function also improved with administration of the MLR glycerolphospholipids [32]. After 12 weeks there was a 35.5% reduction in fatigue ($p < 0.001$), and there was good correspondence between reductions in fatigue and gains in mitochondrial function ($p < 0.001$). By 12 weeks of supplementation mitochondrial function was found to be similar to that of young, healthy adults [32]. However, when subjects were placed on pla-

cebo without their knowledge for an additional 12 weeks, their fatigue and mitochondrial function were intermediate between the initial starting values and those found after eight or 12 weeks on the MLR supplement [32]. Here fibromyalgia patients showed significant reductions in fatigue similar to those found in previous studies with fibromyalgia patients [27].

In addition to fatigue, in the present study we also examined subjects for reductions in widespread pain and gastrointestinal symptoms, common complaints of fibromyalgia patients, as well as changes in QOL indicators. We found significant reductions in pain ($p < 0.001$) and gastrointestinal symptoms ($p < 0.001$) as well as improvements in QOL indicators ($p < 0.001$). The changes in fatigue, pain, gastrointestinal symptoms and QOL indicators were found to be significant on each day of the study ($p < 0.001$). Although there were differences between the responses of males and females in the study, consistent with previously studies, these differences were generally not statistically significant.

Using a mixed-effects statistical model and calculating Marginal and Conditional R^2 values indicated that the data were consistent and occurred with a low degree of variance. Although the number of subjects and time of the study were quite limited, the statistical analysis suggested that increasing the number of participants or the time of the study would be unlikely to change the conclusions. In fact, the results suggested that further reductions in overall scores, pain, fatigue, gastrointestinal symptoms and improvements in QOL were likely to occur with further time on the combination supplement.

Recently a case study with fibromyalgia patients using the MLR glycerolphospholipids (Patented Energy™ wafers) without added controlled-release caffeine demonstrated that individual fibromyalgia patients evaluated in a clinic setting by specialists also showed reductions in pain, fatigue and gastrointestinal symptoms in the same time-frame as the current study (Breeding, P.A. and Nicolson, G.L. in preparation). This indicated that the effects seen here were not due to caffeine alone.

The formulation used in this clinical study contained controlled-release caffeine that delivered a modest dose of caffeine over eight hours. Caffeine in low to moderate doses has been used previously to reduce pain and other symptoms in patients with various diagnoses. Thirty clinical studies involving more than 10,000 patients have been conducted with combinations of drugs and caffeine to assess the value of caffeine as an added adjuvant [34]. For example, the pooled data on use of caffeine to increase the effectiveness of various analgesics compared to the analgesics alone have shown that caffeine can increase the overall relative potency by an estimated factor of 1.41 [34]. Vaeroy *et al.* [35] found that fibromyalgia patients benefited when a low dose of caffeine was added to a combination mixture of carisoprodol and paracetamol (acetaminophen) to reduce pain and other symptoms. When caffeine was in the mixture, the combination was more effective ($p = 0.015$) [35]. Here we did not compare caffeine alone to NTFactor Lipids alone or to the combination of NTFactor Lipids plus con-

trolled-release caffeine, because the caffeine supplement that was under test (Brite Alert™) already contains NTFactor Lipids.

Although the etiology of fibromyalgia remains unclear, some of the possible causes are related to altered pain processing, resulting in malfunction at multiple levels. Some examples include aberrant ion channels, changes in intracellular Ca^{2+} and mitochondrial defects in neuroendocrine and skeletal muscle cell signal processing [36] [37] [38] [39]. Glycerolphospholipids and caffeine might act at these levels, and caffeine is known to be an important modulator of Ca^{2+} release in muscles and neuroendocrine cells [40] [41]. A recent study from our group supports the notion that NTFactor Lipids may act not just by replacement of damaged membrane glycerolphospholipids, but also by modulating and restoring the function of ion channels, intracellular Ca^{2+} and mitochondrial function [42]. The clinical trial results here are consistent with what has been described for the fibromyalgia syndrome etiology and the mechanism of action of NTFactor Lipids® and caffeine.

5. Conclusion

We tested the hypothesis that NTFactor Lipids® plus controlled-release, low-dose caffeine could reduce pain, fatigue, and gastrointestinal symptoms while improving QOL scores in fibromyalgia patients. Within days we found significant self-reported improvements in patients taking the combined supplement. The supplement was safe and effective and significantly reduced pain, fatigue and gastrointestinal symptoms and enhanced QOL scores during the 8-day period of the study. Although the number of patients in the study was limited, the statistical analysis suggested that increased numbers of participants would be unlikely to change the conclusions.

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Disclosures

Garth L. Nicolson and Robert Settineri are part-time consultants to Allergy Research Group, Inc. and Nutritional Therapeutics, Inc. Gonzalo Ferreira and Paul Breeding have no conflicts to disclose.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplementary

Name _____ **Day (circle): 0 1 2 3 8**

IMM COMBINED SYMPTOM SURVEY FORM—FIBROMYALGIA

1.Rate the **level** of your pain at the present moment.

0 1 2 3 4 5 6 7 8 9 10

No pain **Very intense pain**

2.In general, how much does your pain problem interfere with your day-to-day **activities**?

0 1 2 3 4 5 6 7 8 9 10

No interference **Extreme interference**

3.Since the time you developed a pain problem, how much has your pain changed your **ability to work**?

0 1 2 3 4 5 6 7 8 9 10

No change **Extreme change**

___ Check here, if you have retired for reasons other than your pain problem.

4. How much has your pain changed the amount of satisfaction or enjoyment you get from participating in **social and recreational activities**?

0 1 2 3 4 5 6 7 8 9 10

No change **Extreme change**

5. How **supportive or helpful is your spouse** (significant other) to you in relation to your pain?

0 1 2 3 4 5 6 7 8 9 10

Not at all supportive **Extremely supportive**

6. Rate your overall **mood** during the past week.

0 1 2 3 4 5 6 7 8 9 10

Extremely low mood **Extremely high mood**

7. On the average, how **severe** has your pain been during the last week?

0 1 2 3 4 5 6 7 8 9 10

Not at all severe **Extremely severe**

8. How much has your pain changed your **ability to participate** in recreational and other social activities?

0 1 2 3 4 5 6 7 8 9 10

No change **Extreme change**

9. How much has your pain changed the amount of **satisfaction** you get from family-related activities?

0 1 2 3 4 5 6 7 8 9 10

No change **Extremechange**

10. How **worried** is your spouse (significant other) about you in relation to your pain problem?

0 1 2 3 4 5 6 7 8 9 10

Not at all worried **Extremely worried**

11. During the past week, how much **control** do you feel that you have had over your life?

0 1 2 3 4 5 6 7 8 9 10

Not at all in control **Extremely in control**

12. How much **suffering** do you experience because of your pain?

0 1 2 3 4 5 6 7 8 9 10

No suffering **Extreme suffering**

13. How much has your pain changed your **marriage and other family relationships**?

0 1 2 3 4 5 6 7 8 9 10

No change **Extreme change**

14. How much has your pain changed the **amount of satisfaction or enjoyment** you get from **work**?

0 1 2 3 4 5 6 7 8 9 10

No change **Extreme change**

__ Check here, if you are not presently working.

15. Do you have an urgent feeling of need to vomit but it does not occur or have **nausea**?

0 1 2 3 4 5 6 7 8 9 10

None **Very severe**

16. Do you have **vomiting** of mucus and stomach contents or strong unproductive retching?

0 1 2 3 4 5 6 7 8 9 10

None **Very severe**

17. Do you feel **bloated** or congestion of food without prior food intake?

0 1 2 3 4 5 6 7 8 9 10

None **Very severe**

18. Do you have **abdominal cramps or stomach pain** without specific localization?

0 1 2 3 4 5 6 7 8 9 10

None **Very severe**

19. Do you feel that your stomach is **overfilled** soon after starting to rest or not able to finish your meal?

0 1 2 3 4 5 6 7 8 9 10

None **Very severe**

20. Do you have **belching** with acid taste, heartburn, burning sensation in the esophagus or food pipe?

0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

21. Do you experience **discomfort** or sickness combined with the need to **vomit**?
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

22. Do you have loss of **appetite**?
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

23. Do you have an unpleasant feeling or **pain behind the sternum** or breastbone?
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

24. Do you have **pain localized in the upper abdomen** below the sternum or breastbone?
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

25. Rate your **levelof fatigue** on the day you felt **mostfatigued** during the last week.
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

26. Rate your **level of fatigue** on the day you felt **least** fatigued during the last week.
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

27. Rate your **level of fatigue** on the **average** during the last week.
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

28. Rate your level of **fatigueright now**.
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

29. Rate how much in the last week fatigue interfered with your **general level of activity**.
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

30. Rate how much in the last week fatigue interfered with your **ability to bathe and dress.**
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

31. Rate how much in the last week fatigue interfered with your **normal work activity** (includes both work outside the house and housework or work around the home)

0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

32. Rate how much in the last week fatigue interfered with your **ability to concentrate**.
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

33. Rate how much in the last week fatigue interfered with your**relations with other people.**
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

34. Rate how much in the last week fatigue interfered with your**enjoyment of life.**
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

35. Rate how much in the last week fatigue interfered with **yourmood.**
0 1 2 3 4 5 6 7 8 9 10
None **Very severe**

36. Indicate in the last 8 days**how many days** you felt fatigued for any part of the day.
0 1 2 3 4 5 6 7 8
None **Days**

37. Rate **how much of the day**, on the average, you felt fatigued in the last week.
0 1 2 3 4 5 6 7 8 9 10
None **Entire Day**

38. Indicate which of the following best describes the **daily pattern** of your fatigue in the last week.

0	1	2	3	4
None	Worse in morning	Worse in afternoon	Worse in evening	No consistent daily pattern

39. Do you have any difficulty combing your hair?
0 1 2 3 4 5 6 7 8 9 10
None **Very severe difficulty**

40. Can you walk continuously for 20 minutes?
0 1 2 3 4 5 6 7 8 9 10
None **Very severe difficulty**

41. Can you prepare a homemade meal?
0 1 2 3 4 5 6 7 8 9 10

None **Very severe difficulty**

42. Are you able to vacuum, scrub or sweep floors?
0 1 2 3 4 5 6 7 8 9 10
None **Very severe difficulty**

43. Can you lift and carry a bag full of groceries?
0 1 2 3 4 5 6 7 8 9 10
None **Very severe difficulty**

44. Can you climb one flight of stairs?
0 1 2 3 4 5 6 7 8 9 10
None **Very severe difficulty**

45. Can you change bed sheets?
0 1 2 3 4 5 6 7 8 9 10
None **Very severe difficulty**

46. Can you sit in a chair for 45 minutes?
0 1 2 3 4 5 6 7 8 9 10
None **Very severe difficulty**

47. Can you go shopping for groceries?
0 1 2 3 4 5 6 7 8 9 10
None **Very severe difficulty**

Supplementary Figure 1. The signs/symptoms survey form used for this study. Participants were directed to fill out the form in the evening on the indicated days of the study and return the form to the Institute for Molecular Medicine.

Supplementary Table 1. Mean scores and SEM by day for each variable (each question in the symptom survey form).

	Overall		Baseline		Day 1		Day 2		Day 4		Day 6		Day 8	
Variable	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Q1	5.43	2.35	7.62	1.56	6.14	2.01	5.48	2.29	4.81	2.20	4.38	2.29	4.14	1.98
Q2	6.56	2.38	8.10	1.97	7.14	2.20	6.67	2.46	6.10	2.26	5.86	2.43	5.52	2.20
Q3	8.67	1.79	9.43	1.57	9.24	1.61	8.76	1.79	8.52	1.75	8.19	1.78	7.86	1.90
Q4	7.66	2.02	9.19	1.36	8.24	1.79	7.76	1.89	7.14	2.08	7.14	1.88	6.48	2.02
Q5	6.58	2.89	7.19	2.62	7.14	2.65	6.48	2.89	6.43	2.98	6.14	3.20	6.10	3.13
Q6	4.67	1.94	5.95	1.96	5.48	2.02	4.81	2.04	4.19	1.57	3.81	1.57	3.76	1.48
Q7	6.31	1.90	8.05	1.53	7.05	1.66	6.33	1.80	5.95	1.60	5.24	1.61	5.24	1.64
Q8	7.42	2.19	9.00	1.55	8.19	2.04	7.62	2.13	7.00	1.92	6.48	1.86	6.24	2.43
Q9	7.37	2.13	8.71	1.85	8.19	1.86	7.48	2.18	6.95	2.04	6.62	2.06	6.24	1.89
Q10	4.69	2.69	5.86	2.24	5.29	2.57	4.62	2.69	4.38	2.69	4.00	2.85	4.00	2.85
Q11	4.99	2.02	5.76	2.28	5.62	2.22	5.05	2.13	4.81	1.72	4.38	1.72	4.33	1.74

Continued

Q12	6.66	1.93	8.00	1.64	7.52	1.89	6.71	1.82	6.48	1.72	5.76	1.73	5.48	1.63
Q13	7.37	2.35	8.05	2.04	7.38	2.48	7.38	2.38	7.29	2.41	7.05	2.50	7.05	2.40
Q14	8.17	2.01	8.90	1.76	8.71	1.87	8.52	1.91	7.86	2.13	7.57	2.09	7.43	2.01
Q15	2.36	3.40	3.38	3.40	2.52	3.57	2.24	3.35	2.05	3.53	2.05	3.40	1.90	3.36
Q16	0.56	1.26	0.90	1.45	0.76	1.34	0.48	0.87	0.33	0.73	0.33	0.73	0.57	1.96
Q17	2.62	2.93	3.90	2.83	3.14	2.85	2.57	3.12	2.29	2.92	2.05	2.87	1.76	2.81
Q18	2.71	2.70	4.33	2.48	3.33	2.76	2.57	2.66	2.10	2.59	2.05	2.62	1.90	2.57
Q19	2.70	2.91	4.19	2.80	3.95	2.80	2.81	3.03	2.00	2.76	1.76	2.59	1.48	2.62
Q20	2.15	2.84	3.14	3.28	2.71	3.02	2.14	2.83	1.76	2.62	1.62	2.62	1.52	2.56
Q21	1.40	2.79	1.95	3.11	1.52	2.80	1.33	2.92	1.24	2.76	1.19	2.75	1.14	2.67
Q22	3.02	2.89	4.24	2.72	3.62	2.96	2.81	2.96	2.71	2.87	2.52	2.82	2.24	2.83
Q23	2.29	2.57	3.52	3.14	3.00	2.86	2.48	2.56	2.14	2.43	1.48	1.91	1.14	1.71
Q24	2.32	2.56	3.57	2.99	2.90	2.70	2.19	2.14	2.19	2.60	1.67	2.33	1.38	2.16
Q25	7.54	1.92	9.19	0.93	8.57	1.29	7.62	1.80	7.29	1.55	6.57	1.80	6.00	2.00
Q26	5.14	1.87	6.52	1.72	5.52	1.86	5.52	1.75	4.76	1.48	4.57	1.69	3.95	1.77
Q27	6.30	2.08	8.05	1.60	7.29	1.42	6.71	1.55	5.95	1.63	5.29	1.87	4.52	2.20
Q28	5.88	2.71	8.62	1.32	7.10	2.12	5.95	2.31	4.86	2.61	4.62	2.25	4.14	2.65
Q29	6.49	2.17	8.62	1.36	7.48	1.99	6.57	1.96	5.90	1.76	5.48	1.75	4.90	1.92
Q30	4.52	2.86	6.48	2.94	5.48	3.11	4.95	2.46	3.90	2.61	3.38	2.31	2.90	2.19
Q31	6.96	2.29	8.71	1.59	8.00	1.95	7.33	2.18	6.48	1.89	5.86	2.13	5.38	2.22
Q32	6.40	2.17	8.38	1.56	7.38	2.13	6.38	1.94	5.81	1.91	5.57	1.83	4.86	1.71
Q33	6.67	2.33	8.29	1.76	7.38	2.38	7.10	2.17	6.00	2.35	5.71	2.17	5.52	1.97
Q34	7.07	2.40	8.67	1.88	7.90	2.19	7.62	2.13	6.48	2.60	6.05	2.11	5.71	2.17
Q35	6.96	2.31	8.95	1.43	8.14	2.20	7.52	2.29	6.33	1.71	5.52	1.83	5.29	1.85
Q36	8.98	1.78	9.58	1.33	9.40	1.51	9.29	1.88	8.87	1.89	8.51	1.96	8.21	1.84
Q37	7.83	1.91	9.33	1.15	8.71	2.00	8.14	1.85	7.33	1.71	6.90	1.58	6.57	1.60
Q39	2.94	3.06	3.81	3.43	3.38	3.34	3.19	3.36	2.62	2.96	2.43	2.68	2.24	2.55
Q40	4.84	3.48	6.19	3.60	5.57	3.67	5.19	3.79	4.33	3.26	4.19	3.22	3.57	2.98
Q41	4.60	2.75	5.81	3.16	5.00	2.90	4.71	2.67	4.29	2.39	3.95	2.65	3.86	2.52
Q42	5.38	2.52	7.29	2.24	6.24	2.32	5.33	2.54	4.76	2.30	4.43	2.31	4.24	2.19
Q43	4.65	2.64	6.14	2.08	5.19	2.66	4.67	2.67	4.33	2.65	3.86	2.63	3.71	2.57
Q44	4.45	2.81	6.00	2.49	5.05	3.04	4.14	2.80	4.00	2.72	3.95	2.71	3.57	2.64
Q45	4.47	2.36	5.57	2.29	5.05	2.58	4.71	2.26	4.00	2.26	3.86	2.20	3.62	2.16
Q46	4.86	3.00	6.14	2.85	5.43	3.28	4.90	3.02	4.52	2.87	4.19	2.94	3.95	2.77
Q47	4.78	2.63	6.43	2.48	5.24	2.77	4.90	2.70	4.38	2.40	4.00	2.39	3.71	2.33

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