Original Article

Synergistic Effect of Vitamin B12 and Mesenchymal Stem Cells to Alleviate Paclitaxel-Induced Sciatic Neuropathy in Albino Rats Via Down-Regulation of NLRP3 Inflammasome Pathway: Histological and Immunohistochemical Study

Heba Bayoumi[1], Enas Elgendy[1], Samia M. Manawy[2] and Kamal M. Kamal[2]

[1]Department of Histology and Cell Biology, [2]Department of Anatomy and Embryology, Faculty of Medicine, Benha University, Egypt

ABSTRACT

Introduction: Taxanes are a wide group of anticancer drugs. Paclitaxel (PTX) induced peripheral neuropathy is the main long-lasting side effect of paclitaxel. This harmful impact has a significant NLRP3 inflammasome pathway integration. Vitamin B12 combined with BMMSCs may have an alleviating role.

Objectives: This study aims to assess the possible synergistic influence of vitamin B12 and bone marrow mesenchymal stem cells (BMMSCs) to alleviate sciatic neuropathy and define the related anti-NLRP3 inflammasome pathway role.

Study design: About 50 adult albino rats were allocated into 5 groups. Group I (control): no treatments, group II treated with paclitaxel (PTX neuropathy): injected (i.p) with PTX (2.0 mg kg−1) on days 1, 3, 5, and 8, group III (PTX+ vit B12): was treated as group II, then on day 10 rats were injected with vit B12 (10 mg /kg) per every other day (i.m) for 28 days, group IV (PTX+MSCs) as group II, then on day 10, injected (i.v) with a single dose of BMMSCs ($1x10^6$ cells) in 1.0 ml saline. Finally, group V (PTX + vit B12+ MSCs) was treated as group II, then injected with vitamin B12 and BMMSCs at the same doses mentioned before. After sacrificing, sciatic samples were collected, processed, and examined histopathologically, immunohistochemically, and with the electron microscope.

Results: PTX group showed marked histological distortion, congested vasculature, decreased Schwann cells, degenerated fibers, vacuolated axons, upregulated CD68, NLRP3, and caspase-1 immunomarkers. PTX+ vit B12 group showed mild histological improvement, and mild downregulated CD68, NLRP3, and caspase-1 immunomarkers. PTX+MSCs group showed moderate histological improvement and moderate downregulated CD68, NLRP3, and caspase-1 immunomarkers. PTX+ vit B12+ MSCs showed apparent histological improvement, the myelinated fibers appeared nearly normal with apparent downregulated CD68, NLRP3, and caspase1 immunomarkers.

Conclusion: Combined vitamin B12 and BMMSCs therapy synergistically alleviated PTX-neuropathy with obvious NLRP3 inflammasome pathway inhibition.

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Key Words: BMMSCs, neuropathy, NLRP3 inflammasome, paclitaxel, vitamin B12.

Corresponding Author: Heba Bayoumi, MD, Department of Histology and Cell Biology, Faculty of Medicine, Benha University, Egypt, **Tel.**: +20 10 9739 0300, **E-mail:** heba.bayoumi@fmed.bu.edu.eg - histo_h@yahoo.com

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INTRODUCTION

Chemotherapy-induced peripheral neuropathy (CIPN) is considered a severe and long-lasting side effect of many chemotherapeutic agents, such as taxanes, platinum-based compounds, and vinca alkaloids[1]. These neurotoxic chemotherapeutic agents can induce structural damage in the peripheral nerves[2]. One of these chemotherapeutic agents is paclitaxel, which is used to treat people with breast, ovarian, and lung tumors. The pharmaco-toxicological profile of paclitaxel mostly includes hair fall, allergic reactions, diarrhea, bone marrow suppression, and lung inflammation[3].

The existing bottleneck in the therapeutic use of paclitaxel is that it causes advanced and often irreversible damage to the peripheral nervous system in 60–70% of patients getting paclitaxel resulting in abnormal effects on the sensory and motor functions[4]. A key factor in this neuronal damage and neural degeneration is the process of neuroinflammation[5].

It is well known that numerous neuropathological disorders are influenced significantly by the crosstalk between the immune and nervous systems[6]. Macrophages are considered an important cause of inflammation in innate immunity and so, may act as a potential culprit to cellular

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senescence stimulation[7]. During the neuroinflammatory pathway, macrophages and microglia are the most plentiful immune cells activated[8]. Macrophages move to the damaged area and release inflammatory cytokines, starting an inflammatory cascade reaction[9].

The term "inflammasome" describes supramolecular structures in the cytoplasm of stimulated immune cells that trigger the proteolytic activation of proinflammatory caspases, promoting inflammation and other systemic immunological responses[10]. The most thoroughly investigated inflammasome complex among the numerous inflammasomes discovered in mammals is the NOD-like receptor protein 3 (NLRP3) inflammasome[11].

Following activation by inflammatory stimuli, the NLRP3 inflammasome is mostly expressed in immunological and inflammatory cells such as neutrophils, mast cells, and proinflammatory macrophages[12].

The inactive NLRP3 inflammasome is a tripartite protein complex that is put together in response to a wide variety of external pathogens or internal danger signals, which causes the production of pro-inflammatory cytokines and pyroptotic cell death[13]. It is composed of three protein subunits: a sensor molecule (NLRP3), an adaptor protein (ASC), and an effector protein (pro-caspase-1) which functions to switch on the inflammatory process[14,15]. Activated caspase-1 in turn converts pro-IL-1β into active IL-1β, which is then released to enhance more inflammation[16,17].

To date, stem cell technology has undergone great evolution, and it may be used to treat various diseases related to the nerves, lung, heart, and liver[18]. Mesenchymal stem cells (MSCs), a varied subpopulation of stromal stem cells, can be acquired from different tissues, such as bone marrow, dental pulp, peripheral blood, umbilical cord, and adipose tissue[19].

Characterized by little immunogenicity, great anti-inflammatory, immunoregulatory functions and regenerative capacity the use of MSCs in cell treatments and tissue engineering is extremely promising[20,21].

A key aim is to increase the potency and therapeutic advantages of MSCs[22]. Combining MSC therapy with pharmaceutical drugs can be a potential new way to maximize its benefits and minimize its drawbacks.

Based on the above evidence, we aim to target the neuroinflammation of the paclitaxel model of peripheral neuropathy and explore whether vit B12 combined with MSC alleviated neuroinflammation and enhanced axonal regeneration by regulating NLRP3 inflammasome activation, aiming to provide a new avenue for chemotherapy-induced neuropathy treatment.

Drugs

Paclitaxel 100 mg ampoule (6 mg/ml). Product name Taxol was factory-made by Corden Pharma Latina S.P.A. (Sermoneta, Latina, Italy) for Bristol-Myers Squibb Company (Roma, Italy).

Vitamin B12 (Depovit B12®) (AMRIYA Pharmaceuticals Company4, Egypt) as 1 ml ampoules of Hydroxocobalamin 1000 μ/ml.

Animals

For this study, a total of fifty adult albino rats were included. Rats started the study with a body weight of 175-200 grams and were allowed to become accustomed to the experimental animal habitat for 7 days. Throughout the experimental time, the rats were housed in plastic cages (5 per cage) at a temperature of 25–27°C and a humidity of 35–65%, with a 12-h light/dark cycle and fresh air changes/hour, and were allowed free access to rat diet and water. The Research Ethical Committee at the Faculty of Medicine, Benha University approved the research protocol; (NOM: RC 11-8-2023).

BMMSCs isolation and culture[23]

Five rats were anesthetized and were then cervical dislocated to death. We acquired femurs and tibias. After being briefly submerged in 70% isopropanol, dissected femurs and tibias were transferred to PBS media. Afterward, it was put into a 10 cm dish with DMEM. Each bone's ends were severed, and the marrow was extracted and injected into a 50ml falcon tube using a 22G needle and 3ml of DMEM. Each bone received two to three flushing repetitions. To remove the bone fragments and blood clumps, cells were suspended and then run through a 70-micron cell strainer. The obtained cells were spun down at 2000g for 5 minutes, with the supernatant aspirated out. Cells were re-suspended in 25ml of MSC media, which is composed of antibiotic- and 10% FBS-DMEM. Two 10cm culture dishes with a total of 10 ml of cell suspension each were seeded for 1-2 weeks at 37°C and 5% CO2. Every 2 to 3 days, the medium was changed. Analysis of the cultured stem cells was achieved using an inverted microscope provided with a digital camera (Olympus CKX41SF, Tokyo, Japan).

Immunocytochemistry identification of BMMSCs[24]

To identify MSCs, the main antibodies mouse anti-human CD44+ and mouse anti-CD34 were employed as CD markers. The cells were cultured again in multi-well tissue culture plates (8 wells) in MEM supplemented with 20% FBS after being dispersed with trypsin-versene and suspended in growth media. The plates were incubated to develop a monolayer of adherent cells within 3 days, after which the media was removed and the cells were fixed with 4% paraformaldehyde for 10 min. The sections were handled in accordance with the instructions provided by the kit's manufacturer (Santa Cruz Biotechnology, USA), placing them in the humid compartment at room temperature (20–25 °C) at each step. The cells were treated with 1% hydrogen peroxide followed by three 5-minute PBS washes. 1.5% blocking serum was aliquoted and applied to the cell section for an hour before being removed. After being washed for around 30 minutes, 1.2 ml of biotinylated secondary antibody was added, and the 1ry antibody was

incubated for an hour at room temperature or overnight at 4 °C at a ratio of 1:50. Cell section received an addition of 650 l of AB enzyme reagent. Each step before this one ended with washing. Cells were treated with three drops of peroxidase substrate until the appropriate stain intensity appeared. Cell sections were stained with hematoxylin for 5–10 seconds, and then they were promptly rinsed with distilled water. Finally, 1-2 drops of permanent mounting media were added, and L/M analysis was performed

Labeling of BMMSCs with PKH-26 fluorescent dye[25]

BMMSCs were labeled per the manufacturer's instructions with a few minor adjustments using the cell linker (PKH-26) (red fluorescence) (Sigma Chemical). A 2 ml injection of fetal bovine serum stopped the reaction after $2x10^7$ MSCs had been labeled for 4 minutes at room temperature with 2x105 to 2x106 mol/l PKH26. After two washes in 5 ml of Dulbecco's adjusted Eagle's medium, cells were then transferred to animals. Using a fluorescence microscope (Olympus, model: BX50F4, No. 7M03285, Japan), segments of the sciatic nerve from the MSCs-injected groups were studied.

Experimental design

To search the role of NLRP3 inflammasome in the sciatic peripheral neuro-inflammation, and its inhibition by combined vitamin B12 and MSCs therapy, fifty male adult albino rats were allocated into 5 groups. I (Control group), II (PTX-neuropathy group), III (PTX+ vitamin B12 group), IV (PTX+MSCs group), and V (PTX + vit B12+ MSCs group).

Rats of group I: received no treatments.

Peripheral neuropathy was induced in rats of group II by intraperitoneal (i.p) injection of paclitaxel (2.0 mg kg−1) on four alternative days (days 1, 3, 5, and 8)[26].

Rats of Group III: were treated as group II, then were injected on day 10 with vit B12 at a dose of 10 mg /kg/ per every other day intramuscular (i.m) for 28 days[27].

Rats of Group IV: were treated as group II, then on day 10 rats were injected with a single dose of MSCs ($1x10^6$ cells) in 1.0 ml saline intravenously (i.v)[28].

Rats of Group V: were treated as group II, then were injected with vitamin B12 and MSCs at the same doses mentioned before.

The rats were sacrificed by carbon dioxide exposure. Rats of group II were necropsied on day 10, while rats of groups I, III, IV, and V were necropsied on day 39. Sciatic nerve segments (4 cm above the knee joint) were taken and separated into two groups. Samples of one group were fixed in 10% formalin and processed following standard protocol for paraffin block preparation[29]. Sections were handled for histopathological and immunohistochemical examination, while, the other group of samples was fixed in a mixture of glutaraldehyde (2.5%) and paraformaldehyde (2.5%) and processed for electron microscopic examination.

Immunohistochemical study

All steps for immunostained section preparation were done according to standard practice[30]. Depraffinized 5 microns thick sciatic nerve sections were cut and prepared, and tissue sections were treated with 3% hydrogen peroxide for 20 Mins. Washed by PBS, then incubated with mouse anti-CD68 monoclonal antibodies (Kp-1, 1:100; DAKO), anti-NLRP3 (GTX00763 - 1:100; Genetex Co) and anti Caspase-1 ((14F468) NB100-56565 - 1:100; Novus bio Co) overnight at 4C; washed by PBS followed by cultivation with secondary antibody (1:350) HRP Envision kit (DAKO) 15 minutes; rinsing by PBS and incubated with diaminobenzidine about 10 minutes. Washing by PBS then counterstaining with hematoxylin, dehydrated and clearing in xylene then cover slipped for microscopic analysis.

Transmission electron microscopy (TEM) sample preparation

Small parts of sciatic nerves ($1mm^3$) were fixed in a mixture of glutaraldehyde (2.5%) and paraformaldehyde (2.5%) and sent to the Electron Microscope unit (Faculty of Science, Alexandria University, Alexandria, Egypt) for processing and examination. Then samples were post-fixed in osmium tetroxide (1%), dehydrated, and embedded in resin to get semithin sections which were stained with toluidine blue. Selected areas from the semithin sections were cut into ultrathin (80 nm) sections according to the provided methods[31]. Ultrathin sections were examined using a JEM-1400 plus TEM.

IHC analysis

A full HD microscopic camera operated by the Leica application module was used for sciatic slide analysis (Leica Microsystems GmbH, Wetzlar, Germany). At least 4 random non-overlapping fields from each sample were scanned and analyzed for calculation of the mean number of CD68 positive macrophages, and mean area percent of NLRP3 and caspase-1 immunohistochemical expression. Only threshold and brightness modifications were made to the entire image during image manipulation. All fields were examined at magnification power x400. Negative controls are prepared by the same steps with omission of the primary antibody which is replaced by PBS.

Statistical Study

The Statistical Package for Social Science software program, variety 26 (SPSS, Inc., Chicago, USA), was used to evaluate the obtained data. While non-parametric data were displayed as median & range (minimum-maximum), parametric data were provided as mean and standard deviation. For assessments between more than two groups of parametric data, one-way analysis of variance (ANOVA) and post-hoc Tukey were used; for assessments between more than two groups of non-parametric data, Kruskal-Wallis and post-hoc Dunn's were used. Statistical significance was defined as a *P value* less than 0.05.

RESULTS

Identification of BMMSCs

An inverted microscope was used for BMMSCs identified in culture. They appeared as colonies of rounded cells on day 0, and in subculture on day 7 as spindle-shaped cells with multiple processes (Figure 1A). Cells were also identified by immuno-cytochemical reaction for specific CD surface markers of BMMSCs, CD34 is a negative marker and CD44 is a positive marker. The results are shown in (Figure 1B). BMMSCs were detected using chromogen accumulation on the secondary antibody of CD44 and showed a dark brown color, while cells negative for CD34 were still blue (Hematoxylin color only, with no brown stain). These findings indicated bone marrow origin (+ve for CD44) but not hematopoietic origin (-ve for CD34) of the mesenchymal stem cells. After sciatic neuropathy induction, PKH26-labeled BMMSCs were injected in both groups IV and V. Stem cells were tracked in *vivo* by fluorescent microscopy which showed the homing of these labeled cells in sciatic nerve tissues of both groups (Figure 1C).

Combined vit B12 and BMMSCs alleviated sciatic neuropathy in histopathological examination of sections from experimental groups

In H&E stained longitudinal sections of sciatic nerves, the control group showed normal histological morphology including normal axons, myelin sheaths, and Schwann cells (Figure 2a). After PTX toxicity, sciatic sections showed distorted histological architecture, with marked axonal degeneration, vacuolated fibers, marked congested blood vessels, inflammatory cell infiltrate, and myelin debris were seen (Figures 2b,c). Group III (PTX+ vit B12) showed slightly improved histological appearance involving less degenerated fibers and less congested blood vessels (Figure 2d). Group IV (PTX+MSCs) showed moderately improved histological morphology. Some areas showed proliferated Schwann cells. Büngner bands were detected as large denervated Schwann cells arranged in a linear pattern indicating a sign of regeneration (Figure 2e). Group V (PTX+ vit B12 +MSCs) showed apparent improved histological morphology. Most axons were intact surrounded by normal Schwann cells, except for some focal areas of vacuolization (Figure 2f).

Combined vit B12 and BMMSCs down-regulated CD68, NLRP3 and caspase-1 inflammasome pathway immune markers in experimental groups

In the sciatic sections of the control group, we observed minimal CD68/ NLRP3/ caspase-1 immunoexpression (Figures 3a, 4a, 5a) respectively. Activation of the inflammasome-NLRP3 pathway is involved in PTX-induced sciatic neuropathy. The average number of CD68 positive proinflammatory macrophages was increased, and the mean areas of NLRP3 and caspase-1 expression were greater compared to the control group (Figures 3b, 4b, 5b). Immunoexpression of the three markers was identified as cytoplasmic brown color. Treatment with each vit B12 and BMMSCs separately slightly down-regulated the immunoexpression of these inflammasome pathway markers indicating cessation of inflammation in these groups (Figures 3c, 4c, 5c) and (Figures 3d, 4d, 5d) respectively. On the other hand, CD68/ NLRP3/ caspase-1 positive immunoreactions were sparsely observed in (PTX+ vit B12+ MSCs) group (Figures 3e, 4e, 5e) compared to the pathological group and groups treated with each therapy alone, indicating higher cessation of inflammation.

Quantitative Analysis

To determine the exact expression of pathological changes of CD68/NLRP3/Caspase-1 inflammasome markers, four pictures were photographed under x400 magnification for each slide to encompass the immunoexpression. The statistical analysis was represented in (Figures 3f, 4f, 5f) for CD68, NLRP3, and caspase-1 respectively. In the PTX-neuropathy group, CD68, NLRP3, and Caspase-1 were highly significantly upregulated ($p<0.05$) compared to the control group. This up-regulation was slightly reversed in groups treated with vitamin B12 and BMMSCs separately compared to the pathological group ($p<0.05$). Higher significant downregulation of inflammasome pathway markers was observed in group treated with combined therapy compared to other treated groups ($p<0.05$) (Table 1, Table 2).

Combined vit B12 and BMMSCs alleviated sciatic ultrastructure damage in experimental groups

Semithin sections

Moreover, in toluidine blue stained semithin sections, the control group showed multiple axons variable in shape and size, surrounded by compact myelin sheaths and embedded in connective tissue endoneurium (Figure 6a). After PTX treatment most axons became necrotic and surrounded by degenerated myelin sheaths with splitting of their lamellae. The endoneurium was filled with necrotic debris. Mononuclear cellular infiltrate and large congested blood vessels were observed (Figure 6b). In vit B12 treated group the axons were slightly improved in structure compared to the neuropathy group, but many myelin sheaths showed vacuolations. Few myelinated axons were necrotic (Figure 6c). The MSCs-treated group showed most axons with their myelin sheaths appeared more or less than normal. Few axons showed irregular myelin sheaths with invaginations and evaginations and thin myelin sheaths (Figure 6d). Combined vit B12+MSCs group showed apparent improved histological morphology. Most axons were intact with their myelin sheaths appearing nearly normal (Figure 6e).

Ultrathin sections

The control group (Figure 7) showed multiple myelinated axons surrounded by endoneurium connective tissue. Schwann cells wrapped around myelinated axons with euchromatic nuclei, rough endoplasmic reticulum,

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الملخص العربى

التأثير التآزري لفيتامين ب١٢ والخلايا الجذعية الوسيطة للتخفيف من الاعتلال العصبي الوركي الناجم عن باكليتاكسيل في الجرذان البيضاء عن طريق تثبيط المسار الالتهابي NLRP3 دراسة هستولوجية و هستوكيميائيه مناعية

هبة بيومي[١]، ايناس الجندي[١]، سامية محمود مناوي[٢]، كمال مصطفى كمال[٢]

[١]قسم الأنسجة و بيولوجيا الخلية، [٢]قسم التشريح والأجنة، كلية الطب البشري، جامعة بنها، مصر

الخلفية: التاكسانات هي مجموعة واسعة من الأدوية المضادة للسرطان. ويعتبر الاعتلال العصبي المحيطي هو التأثير الجانبي الرئيسي للباكليتاكسل و المقيد للجرعة طويله الأمد. يحتوي هذا التأثير الضار على تأثير واضح في المسار الالتهابي NLRP٣. و قد يكون لفيتامين ب ١٢ مع الخلايا الجذعية الوسيطة المشتقه من نخاع العظام دور مخفف.

هدف البحث: الهدف من هذه الدراسة هو تقييم التأثير التآزري المحتمل لفيتامين ب١٢ و الخلايا الجذعية الوسيطة المشتقة من نخاع العظام في تخفيف الاعتلال العصبي الوركي وتحديد التأثير المضاد للمسار الالتهابيي NLRP٣

المواد و الطرق المستخدمة: تم توزيع حوالي ٥٠ فأرًا أبيضًا بالغًا على خمسة مجموعات. مجموعة (التحكم) لم تتلقى أي علاج، المجموعة الثانية (مجموعة الاعتلال العصبي بواسطة الباكليتاكسل): تم حقن الفئران داخل الصفاق بعقار الباكليتاكسل (٢,٠ مجم كجم−١) في الأيام ١ و٣ و٥ و٨، المجموعة الثالثة (باكليتاكسل + فيتامين ب ١٢) عولجت مثل المجموعة الثانية ، ثم في اليوم العاشر، تم حقن الفئران بفيتامين ب ١٢ (١٠ ملغم / كغم) كل يومين داخل العضل لمدة ٢٨ يومًا، المجموعة الرابعة (الباكليتاكسل + الخلايا الجذعية الوسيطة) عولجت مثل المجموعة الثانية، ثم في اليوم العاشر، تم حقن الفئران عن طريق الوريد بجرعة وحيدة من الخلايا الجذعية الوسيطة المشتقة من نخاع العظام (١x١٠٦خلية) في ١ ملم من محلول الملح. وأخيراً, المجموعة الخامسة (الباكليتاكسل + فيتامين ب ١٢+ الخلايا الجذعية الوسيطة) عولجت مثل المجموعة الثانية ، ثم تم حقنها بفيتامين ب ١٢و الخلايا الجذعية الوسيطة بنفس الجرعات المذكورة سابقاً. تم ذبح الفئران وجمع عينات من الأنسجة العصبية الوركية ومعالجتها وفحصها نسيجيا ومناعيا وكيميائيا وتحت المجهر الإلكتروني.

النتائج: أظهرت مجموعة الباكليتاكسل تشوهًا نسيجيًا ملحوظًا، واحتقان بالأوعية الدموية، وانخفاض عدد خلايا شوان، وتدهور في الألياف، وفراغات في الخلايا، وزياده في المؤشرات المناعية لبروتينات ضد CD٦٨ وNLRP٣ و caspase-١ . وأظهرت مجموعة باكليتاكسل + فيتامين ب ١٢ تحسنًا نسجيًا بسيطا، وانخفاض بسيط في المؤشرات المناعية ضد CD٦٨ وNLRP٣ وcaspase-١. أظهرت مجموعة الباكليتاكسل مع الخلايا الجذعية الوسيطة تحسنًا نسيجيًا معتدلًا و انخفاض معتدل للمؤشرات المناعية ضد CD٦٨ وNLRP٣ وcaspase-١. و أظهرت مجموعه الباكليتاكسل و فيتامين ب ١٢ و الخلايا الجذعية الوسيطة تحسنًا نسجيًا واضحًا، وبدت أغلفة الميالين طبيعية إلى حد ما مع وجود انخفاض واضح للمؤشرات المناعية ضد CD٦٨ وNLRP٣ وcaspase١.

الاستنتاج: يمكن للعلاج المشترك بواسطة فيتامين ب ١٢ والخلايا الجذعية الوسيطة أن يخفف بشكل تأزري من الاعتلال العصبي الناجم عن الباكليتاكسل مع تثبيط واضح المسار الالتهابي NLRP٣.