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Intermittent pulses of methylprednisolone with low-dose prednisone attenuate lupus symptoms in B6.MRL-Fas^{lpr}/J mice with fewer glucocorticoid side effects

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ABSTRACT

Glucocorticoids (GCs) are potent anti-inflammatory and immunosuppressant medications and remain the cornerstone of systemic lupus erythematosus (SLE) therapy. However, ongoing exposure to GCs has the potential to elicit multiple adverse effects. Considering the irreplaceability of GCs in SLE therapy, it is important to explore the optimal regimen of GCs. Here, we compared the long-term efficacy and safety of pulsed and oral GC therapy in a lupus-prone mouse model. Mice were grouped using a randomized block design. We monitored survival rates, proteinuria, serum autoantibodies, and complement 3 (C3) levels up to 28 weeks of age, and assessed renal damage, bone quality, lipid deposition in the liver and marrow, glucose metabolic parameters, and levels of hormones of the hypothalamic-pituitary-adrenal (HPA) axis. Finally, we explored the mechanisms underlying the superior efficacy of the pulse regimen over oral prednisone regimen. We found that both GC regimens alleviated the poor survival rate, proteinuria, and glomerulonephritis, while also reducing serum autoantibodies and increasing the level of C3. The pulsed GC regimen showed less resistance to insulin, less suppression of the HPA axis, less bone loss, and less bone marrow fat deposition than the oral GC regimen. Additionally, GC-induced leucine zipper (GILZ) was significantly overexpressed in the GC pulse group. These results suggest that the GC pulse regimen ameliorated symptoms in lupus-prone mice, with fewer side effects, which may be related to GILZ overexpression. Our findings offer a potentially promising GC treatment option for SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease with a complex pathogenesis and high heterogeneity [1]. Alongside an improved understanding of SLE, its treatment and the prognosis have greatly improved in recent decades [2]. However, SLE cannot be cured completely. The relapsing and remitting disease course necessitates the long-term treatment of SLE [3]. Glucocorticoids (GCs) are still the first-line treatment for SLE [4]. A daily high-dose oral GC regimen during the induction period remains the most common treatment for moderate to severe SLE, as recommended by the consensus treatment guidelines of international panels [5–8]. However, long-term use of daily high-dose oral GCs can cause serious side-effects, including osteoporosis, skeletal growth inhibition, weight gain, abnormal glucose metabolism, and even death [9]. Considering the potent anti-inflammatory effects and the irreplaceability of GCs as a treatment for SLE, it is necessary to explore a reasonable GC treatment regimen.

Previous studies have shown that short-term GC pulse therapy can rapidly regulate the immune response and simultaneously activate monocytes [10]. Limited observational cohort studies have suggested that GC pulse therapy is an independent predictor of remission of autoimmune disease [11,12]. Recently, attention has been paid to the use of GC pulse therapy for SLE. Ruiz-Arruza et al.[13] found that methylprednisolone (MP) pulses were not associated with accrual

Abbreviations: ANOVA, Analysis of variance; CRH, Corticotropin-releasing hormone; ELISA, Enzyme-linked immunosorbent assay; GILZ, GC-induced leucine zipper; H&E, Hematoxylin and eosin; HPA, Hypothalamic-pituitary-adrenal; IOD, Integral optical density; ITT, Insulin tolerance tests; LN, Lupus nephritis; PAS, Periodic acid-Schiff; SD, Standard deviation; SLE, Systemic lupus erythematosus; TRAP, Thrombin receptor agonist peptide; WT, Wild-type; Pred, Prednisone; MP, Methylprednisolone.

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damage in patients with SLE. Subsequent study found that the administration of repeated MP pulses combined with immunosuppressant enabled reduction of the dose of oral prednisone (pred) and enhanced the clinical response of lupus nephritis to the combination therapy compared with the standard regimen [14]. Recent research supported that MP pulses contribute to rapid and prolonged control of lupus activity accompanied by a reduction of the dosage of oral prednisone [15]. Pulse administration of GCs showed a more rapid onset of action and higher bioavailability with a short half-life in vivo [16], suggesting that GC pulse regimen may have less impact on patients, and may be a better treatment regimen than the oral GC regimen [17]. However, few studies have systematically compared the safety and efficacy of GC pulse and oral GC therapy for SLE. Therefore, this study compared the long-term efficacy and safety of both GC therapies in B6.MRL-Fas^{lpr}/J autoimmune-prone mice receiving equal doses of each GC regimen.

2. Materials and methods

2.1. Mice and experimental protocol

Female B6.MRL-Fas^{lpr}/J mice and C57BL/6 J wild-type (WT) mice aged 6–7 weeks were obtained from the Model Animal Research Center of Nanjing University (Medical Animal Experimental Center of the Nanjing Military Region of China) and housed under specific pathogen-free conditions. All the experiments were carried out in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals. And approved by the Subcommittee on Research Animal Care of the First Hospital of Jilin University (Approval No. 20210034).

All treatments began at 12 weeks of age. Thirty female B6.MRL-Fas^{lpr}/J mice weighing 22–28 g were randomly divided into the following four groups using a randomized block design [18]: 1) The mycophenolate mofetil (MMF) treated group (MMF group, n=7); 2) Repeated MP pulses combined with low-dose oral prednisone treatment group (MP pulse group, n=7); 3) Daily oral prednisone treatment group (oral pred group, n=7); and the 4) Daily saline group (saline group, n=9), which served as the untreated control group. Additionally, six age matched female C57BL/6 J wild-type (WT) mice were used as the blank controls.

Oral GCs have been shown to be effective in murine models of lupus, although the doses given vary [19–21]. To better simulate the current oral GC regimen used in clinical practice, prednisone (Roche, Basel, Switzerland) was administered daily by gavage started at 2 mg/kg and reduced by 0.4 mg/kg every three weeks until the dose reached 0.2 mg/kg, in the oral pred group. The average GC dose was 1.13 mg/kg/d (equivalent to prednisone) in this group. Additionally, MMF (33.3 mg/kg, Roche, Basel, Switzerland) [19] was added as a common GC-sparing agent, following current guidelines. MP, which is often used in pulse therapy owing to its high bioavailability and wide distribution [22], was the type of GC chosen for the pulse regimen in this study. The frequency and dose of MP pulses were as reported in a previous study [14]. To ensure comparability of the two GC regimens, the oral prednisone dose was equal during the maintenance period, and that the total GC dose was equal during the experiment. Therefore, the MP pulse group received six weekly intravenous injections of MP (15 mg/kg; Pfizer, Puurs, Belgium). Prednisone (0.2 mg/kg/d; Roche, Basel, Switzerland) was administered daily by gavage during the interval and maintenance periods of the MP pulses. The average GC dose in this group was 1.19 mg/kg/d (equivalent to prednisone). The dosage and administration of MMF were the same as for the oral pred group. The detailed strategies are shown in Fig. 1. MMF at 33.3 mg/kg was administered daily by gavage in the MMF group. Saline was injected intravenously into the mice in the saline, MMF, and oral pred groups in accordance with the same schedule. All treatments were administered for 16 weeks. The body weights and fasting blood glucose levels of all mice were monitored using an electronic scale and a glucometer (Sinocare, Hu nan, Changsha, China), respectively.

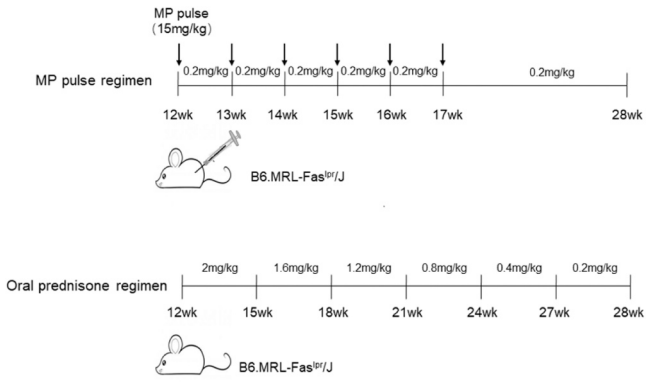

Fig. 1. Working scheme of MP pulse group and oral pred group. In the oral pred group, prednisone was started at a dose of 2 mg/kg/d and then tapered regularly to 0.2 mg/kg/d (equivalent to 5–7.5 mg/d for humans, a dose that is now widely considered to have few toxicity). We designed the MP pulse regimen on the basis of ensuring the same total GC dosage in the two groups. In MP pulse group, 15 mg/kg methylprednisolone was given intravenously once a week for a total of 6 times. For the other days, 0.2 mg/kg/d prednisone was given orally.

2.2. Serum and urine biochemical parameters

Blood and urine samples were collected 12 weeks before and 28 weeks after treatment. Serum and urine samples were then stored at −80 ℃ until they were analyzed. The levels of serum C-telopeptide of type I collagen (CTX-1) and osteocalcin (OCN) at 28 weeks and those of anti-double stranded DNA (dsDNA), complement 3 (C3), adrenocorticotropic hormone (ACTH), corticotropin-releasing hormone (CRH), and Immunoglobulin G (IgG) at 12 and 28 weeks were measured using enzyme-linked immunosorbent assay (ELISA) kits (MEIMIAN, Jiang Su, China). In addition, urine albumin concentration (at 28 weeks) was measured using a mouse albumin ELISA kit (MEIMIAN, Jiang Su, China).

2.3. Organ index and insulin tolerance tests

At 16 weeks' post-treatment, all surviving mice were euthanized by cervical dislocation. The spleen and adrenal glands were removed and weighed. The organ index was calculated as organ weight/body weight, as previously described [23]. The 6-h fasted mice underwent insulin tolerance tests (ITT; 0.75 U insulin/kg) after 16 weeks of treatment [24]. Blood was collected from the tail, and glucose concentrations were measured at the indicated times between 0 and 120 min.

2.4. Scores of lymph nodes in mice

B6.MRL-Fas^{lpr}/J mice have enlarged cervical and axillary nodes; therefore, we harvested the largest cervical and axillary nodes for evaluation. Lymph nodes were scored as follows: 0, negative; 1, milder enlargement on one side; 2, milder enlargement on bilateral lymph nodes; 3, enlargement (not influencing action); and 4, positive (large/firm, and influencing action) [25]. The ratings were performed by two independent researchers.

2.5. Renal histological analysis

Kidney tissues were collected from the mice, fixed in 4 % formaldehyde and embedded in paraffin for histopathology. The slides were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Masson's trichrome and examined under a light microscope. All microscopic evaluations were performed by a pathologist. Glomerulonephritis was scored semi-quantitatively on a 4-point scale independently and blindly by a pathologist: 0–4 represented 0, 1–19, 20–50, and

51–75, and > 75 % affected glomeruli, respectively. A score of 0 indicates a healthy condition; 1 represents mild focal disease; 2 represents moderate focal disease; while 3 and 4 represent severe glomerulonephritis [26]. At least 50 glomeruli were evaluated per mouse.

2.6. Bone histological analysis

The right femur was completely separated, fixed in 4 % formaldehyde at room temperature, and decalcified in 10 % ethylenediaminetetraacetic acid after weighing. The samples were then embedded in paraffin, sectioned, and stained with H&E. The sections were also stained with Oil Red O to analyze fat deposits (bone marrow) [27]. Then, the samples were stained with the thrombin receptor agonist peptide (TRAP) reagent (Sigma-Aldrich, St Louis, MO, USA) to measure osteoclast activity. Five different images were randomly collected from each sample for final analysis. Image-Pro Plus v 6.0 software (Media Cybernetics Inc, Bethesda, MD, USA) was used to analyze the images. Trabecular bone area, bone volume/tissue volume (BV/TV), and trabecular number (Tb. N) were measured as parameters of trabecular bone microstructure [28]. The concentration of TRAP-positive cells (TRAP% area) was quantified relative to the total trabecular bone surface [29].

2.7. Immunohistochemistry

Paraffin-embedded femurs were sectioned and placed on slides coated with 3-aminopropyltriethoxysilane. After deparaffinization and rehydration, the sections were immersed in a citrate buffer (PH=6.4). Endogenous peroxidase activity was blocked by incubation of the slides in 3 % H_20_2, non-specific binding sites were blocked with goat serum (10 %). Then, the sections were blocked with primary rabbit polyclonal antibody against OCN (1: 200 dilution, Servicebio, Wuhan, China) at 4℃ overnight. After being rinsed with PBS three times followed by incubation with secondary antibody enzyme-labeled goat anti-rabbit IgG (1: 300 dilution, Servicebio, Wuhan, China) for 50 min, sections were stained with diaminobenzidine (DAB), counterstained with hematoxylin for 5 min, dehydrated, clarified, and mounted. The region of interest was the distal femur growth plate and metaphysis located 0–2 mm distal to the epiphyseal junction of the growth plate [21]. Images of the region of interest in each sample were captured at 200× magnification. Five different images were randomly collected per sample and subjected to final analysis (Image-Pro Plus v6.0 software). OCN expression was evaluated using integral optical density (IOD) values of the images.

2.8. Reserve transcription quantitative polymerase chain reaction

Total RNA was isolated from the liver and bone (with bone marrow) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Genomic DNA was removed and complementary DNA (cDNA) was reverse-transcribed using Trans-Script All-in-one First-Strand cDNA Synthesis SuperMix for quantitative polymerase chain reaction (qPCR; TransGen Biotech, Beijing, China). qPCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using the FastStart Universal SYBR-Green Master Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. The expression level was normalized against that of β-actin. Relative messenger RNA (mRNA) levels were calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences used for GC receptor alpha (GRα), GC-induced leucine zipper (GILZ), peroxisome proliferators-activated receptor (PPAR)-γ_1, PPAR-γ_2, and β-actin are presented in Table 1.

2.9. Statistical analysis

The results and measurements were expressed as mean ± standard deviation (SD). Between-group comparisons were performed using two-tailed t-tests. Continuous outcomes were compared among three groups using analysis of variance (ANOVA) or Kruskal-Wallis tests. Multiple-group comparisons were performed using ANOVA, followed by Tukey's method. Survival rates were analyzed using the Kaplan-Meier method and compared using the log-rank test. GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA) and SPSS software (version 16.0 SPSS Inc., Chicago, IL, USA) were used for statistical analyses. Statistical significance was set at $p<0.05$.

Table 1
The quantitative PCR (qPCR) primers.

Primer name	Primer sequence
β-actin	F: 5' TTCAACACCCCAGCCATG 3' R: 5' CCTCGTAGATGGGCACAGT 3'
Fasn	F: 5' CTCCTGAAGCCGAACACCTCTG 3' R: 5' AGCGACAATATCCACTCCCTGAATC 3'
GRα	F: 5' CTGCCTGGTGTGCTCCGATG 3' R: 5' TTGTGCTGTCCTTCCACTGCTC 3'
PPARγ$_1$	F: 5' GCCAAGGTGCTCCAGAAGATGAC 3' R: 5' GTGAAGGCTCATGTCTGTCTCTGTC 3'
PPARγ$_2$	F: 5' GCCAAGGTGCTCCAGAAGATGAC 3' R: 5' GTGAAGGCTCATGTCTGTCTCTGTC 3'
GILZ	F: 5' TGTGAGAGAGGAGGTGGAGGTC 3' R: 5' CAGCGTCTTCAGGAGGGTGTTC 3'

F: forward, R: reverse.

3. Results

3.1. MP pulse and oral prednisone regimens show similar efficacy in lupus-prone mice

To compare the therapeutic effect of the MP pulse regimen with that of the oral pred regimen, we used B6.MRL-Fas^{lpr}/J mice, a spontaneous murine SLE model (referred to as lupus-prone mice) that shows systemic autoimmunity symptoms by producing autoantibodies [30]. In addition to bone mass and fat accumulation in the bone marrow, B6.MRL-Fas^{lpr}/J mice developed clinical phenotypes of SLE at 12 weeks, with more severe symptoms at 28 weeks (Supplement Fig. 1).

Serum levels of anti-ds DNA, IgG, and C3 were examined before (12 weeks of age) and after (28 weeks of age) treatment. At 28 weeks, the anti-ds DNA levels were lower in all three treatment groups and higher in the saline control group than at 12 weeks ($p < 0.05$; Fig. 2A). The serum levels of IgG increased in all groups at 28 weeks; however, the MP pulse and oral pred groups showed a smaller increase than the saline and MMF groups ($p < 0.05$; Fig. 2B). In contrast, serum C3 levels increased in the treatment groups and decreased in the saline control group after treatment ($p < 0.05$; Fig. 2C). However, during the 16-week treatment, no significant differences in serum anti-ds DNA, IgG, and C3 levels were observed between oral pred and MP pulse groups (Fig. 2A-C).

To evaluate lymphoproliferation and splenomegaly, the lymph node scores and spleen indices were examined at 28 weeks of age. Compared with the saline control group, MMF, oral pred and MP pulse groups showed significantly decreased spleen indices (p=0.0496, p=0.0041, and p=0.0023, respectively; Fig. 2D) and lymph node scores (p=0.0407, p=0.0071, and p=0.0021, respectively; Fig. 2E). However, there were no significant differences in lymph node scores and spleen indices between the oral pred and MP pulse groups (Fig. 2D, E).

Generally, high mortality rates have been observed among B6.MRL-Fas^{lpr}/J mice [31], consistent with the findings of the present study. Approximately 50 % of the mice in the saline group died before the end of treatment (44.4 %). Two of the seven mice in both the MMF and oral pred groups died. Only one of the seven mice treated with the MP pulse regimen died (Fig. 2F). The survival rate in the MP pulse group was similar to that in the oral pred group; however, a better survival rate was observed in the MP pulse group than in the saline group, although the differences were not statistically significant (p=0.11). These results suggest that MP pulse and oral pred regimens have similar SLE-ameliorating effects.

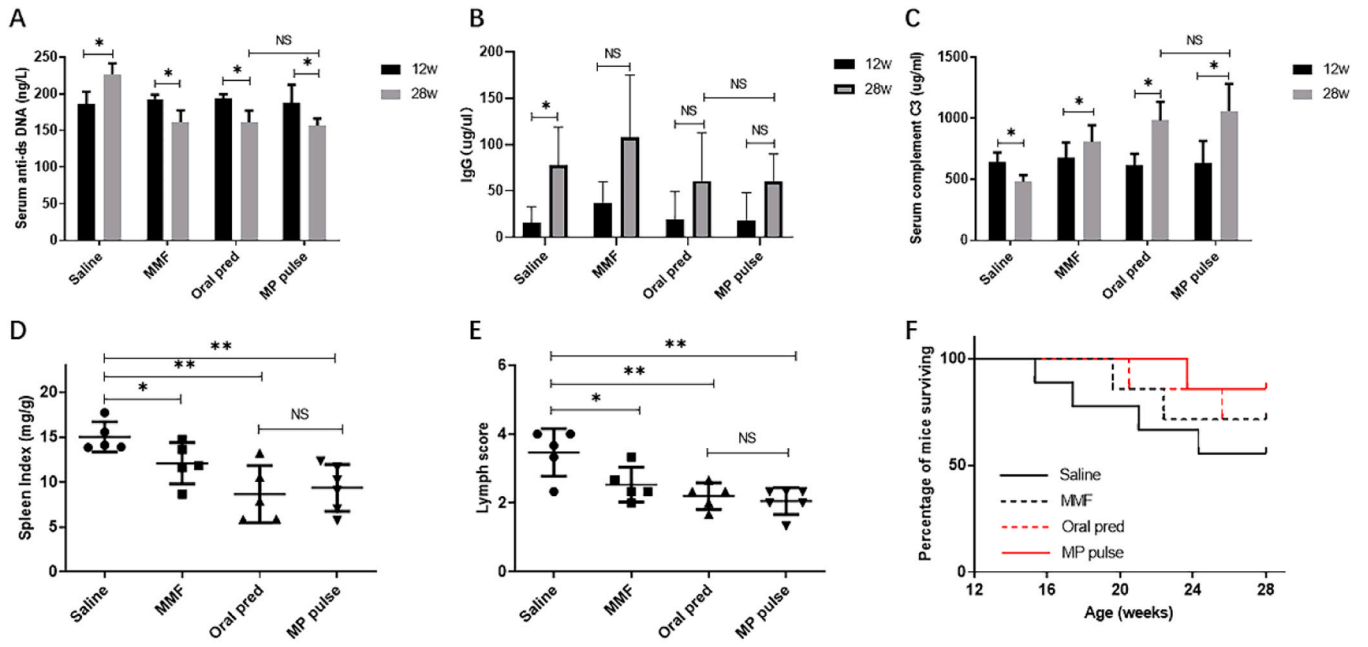

Fig. 2. MP pulse regimen and oral pred regimen show similar therapeutic benefits in B6.MRL-Fas^{lpr}/J mice. 12-week old B6.MRL-Fas^{lpr}/J mice were treated with saline, MMF alone, oral pred regimen or MP pulse regimen. After 16-week treatment, samples from mice that were still alive at the end of the experiment were analyzed (n=5–6 per group). Serum concentrations of anti-ds DNA antibody (A), IgG (B) andC3 (C)at 12-week and 28-week time points. (D) Spleen index of B6.MRL-Fas^{lpr}/J mice. (E) Scores of lymph nodes. Samples were analyzed independently by three experimenters and average scores were used for analysis. (F) Kaplan-Meier survival curves of B6.MRL-Fas^{lpr}/J mice that received different treatment (Log-rank test). Results are expressed as mean ± SD. * $p<0.05$, ** $p<0.005$, *** $p<0.001$. NS represents no significant differences.

3.2. Both MP pulse and oral prednisone regimens ameliorate nephritis in lupus-prone mice

Nephritis is one of the most severe complications of SLE. B6.MRL-Fas^{lpr}/J mice developed renal dysfunction. We evaluated renal involvement, including proteinuria and renal histopathology in the lupus-prone mice. The MMF, oral pred, and MP pulse groups had a significantly lower incidence of proteinuria than the saline group at the end of the experiment ($p=0.0003$, $p=0.0001$, and $p<0.0001$, respectively), whereas the incidence of proteinuria in the oral pred and MP pulse groups did not differ significantly (Fig. 3B). Renal histology analysis showed that both the glomerular basement and mesangial membranes of the B6.MRL-Fas^{lpr}/J mice exhibited active proliferation and inflammation (Fig. 3A). Compared with the oral pred and MP pulse groups, 60 % of mice in the saline group and 40 % of mice in the MMF group had a higher incidence of damaged glomeruli (scores of 3 and 4).

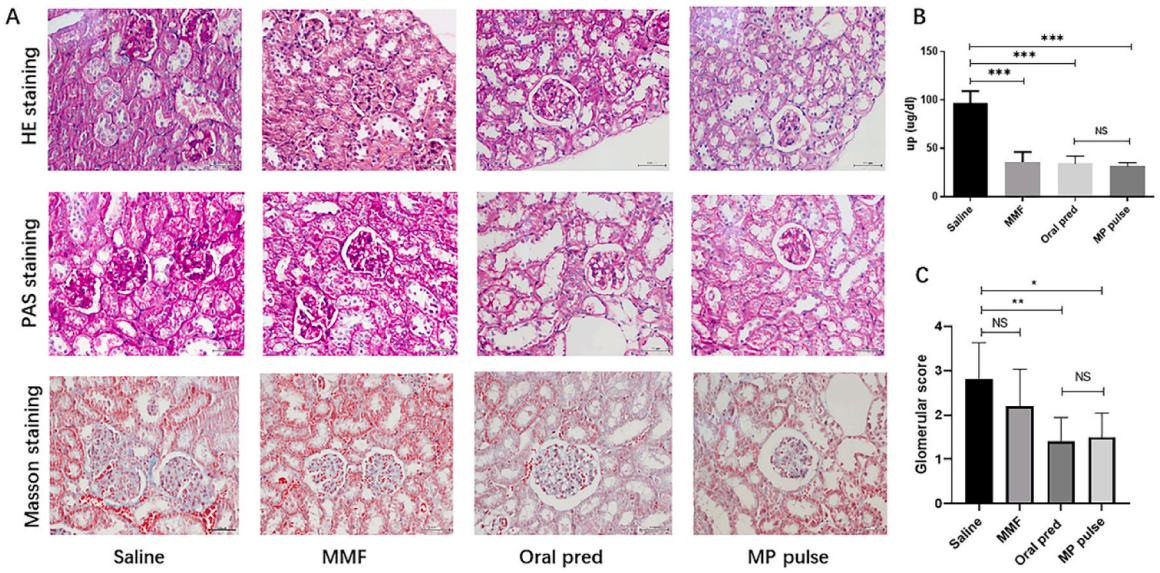

Fig. 3. The evaluation of kidneys from different treatment groups. (A) Representative histological sections of kidney from 28-week-old B6.MRL-Fas^{lpr}/J mice that were treated with saline (n=5), MMF (n=5), MP pulse regimen (n=6) and oral pred regimen (n=5). The sections were stained with H&E (the top panel), PAS (the middle panel), Masson's trichrome (the bottom panel). Original magnifications × 200. Scale bar=100μm. (B) Urine proteinuria in 28-week-old B6.MRL-Fas^{lpr}/J mice of each group (n=4 per group). (C) The glomerular damage score in 28-week-old B6.MRL-Fas^{lpr}/J mice treated with saline (n=5), MMF (n=5), MP pulse regimen (n=6) and oral pred regimen (n=5). Results are expressed as mean ± SD. * $p<0.05$, ** $p<0.005$, *** $p<0.001$. NS represents no significant differences.

In contrast, the MP pulse and oral pred groups exhibited mild or moderate glomerulonephritis, demonstrating significantly lower glomerular damage scores than the saline group (p=0.0126, p=0.014, respectively; Fig. 3C). There was no significant difference in glomerular damage score between the MP pulse and oral pred groups (Fig. 3C). These results further support the efficacy of the MP pulse regimen for treating SLE, including lupus nephritis, which is similar to that of the oral pred regimen.

3.3. *The detrimental effects of MP pulse and oral prednisone regimens on systemic metabolism in lupus-prone mice*

We next compared the side effects of the MP pulse regimen with those of the oral pred regimen. High doses of GCs are frequently associated with metabolic disorders, including weight gain, adipose tissue redistribution, and insulin resistance [32]. First, we monitored the body weight of each group of lupus-prone mice. Unlike in humans, the administration of GCs causes weight loss in mice [21]. Our results showed that body weights in the saline group increased over time, with a slow increase after 24 weeks of age, and a slight reduction at 28 weeks of age. The MMF group showed a similar weight gain trend. In contrast, a decrease in body weight was observed in the MP pulse and oral pred groups (Fig. 4A). At 28 weeks of age, the body weights in the MP pulse and oral pred groups were significantly lower than those in the saline group (p <0.05, Fig. 4A). However, no significant difference was observed between body weights of mice in the MP pulse and oral pred groups.

Long-term exposure of GCs potentiates visceral fat accumulation, with the liver being the organ commonly involved in lipid deposition. Therefore, we assessed liver steatosis in each group of lupus-prone mice, primarily comparing the MP pulse and oral pred groups. H&E staining of the liver tissue revealed hepatocyte necrosis formation and eosinophilic bodies, granulocyte infiltration, and liver cell degeneration in the saline group, while the three treatment groups showed improvement in the above (Fig. 4B). Notably, lipid deposition were similar in the MP pulse and oral pred groups (Fig. 4B). Contrary to expectations, neither group exhibited increased lipid deposition compared with the saline and MMF groups (Fig. 4B). We further analyzed the expression of PPAR-γ_1, a marker of adipogenesis, and fatty acid synthase (Fasn), a marker of lipogenesis, in liver lysates. No differences in PPAR-γ_1 and Fasn mRNA expression were observed between the four groups (Supplementary Fig. 2).

To investigate the effects of the MP pulse and oral pred regimens on glucose metabolism, we monitored the fasting blood glucose level of each group of B6.MRL-Fas^{lpr}/J mice during treatment. The fasting blood glucose level in the oral pred group gradually increased and began to

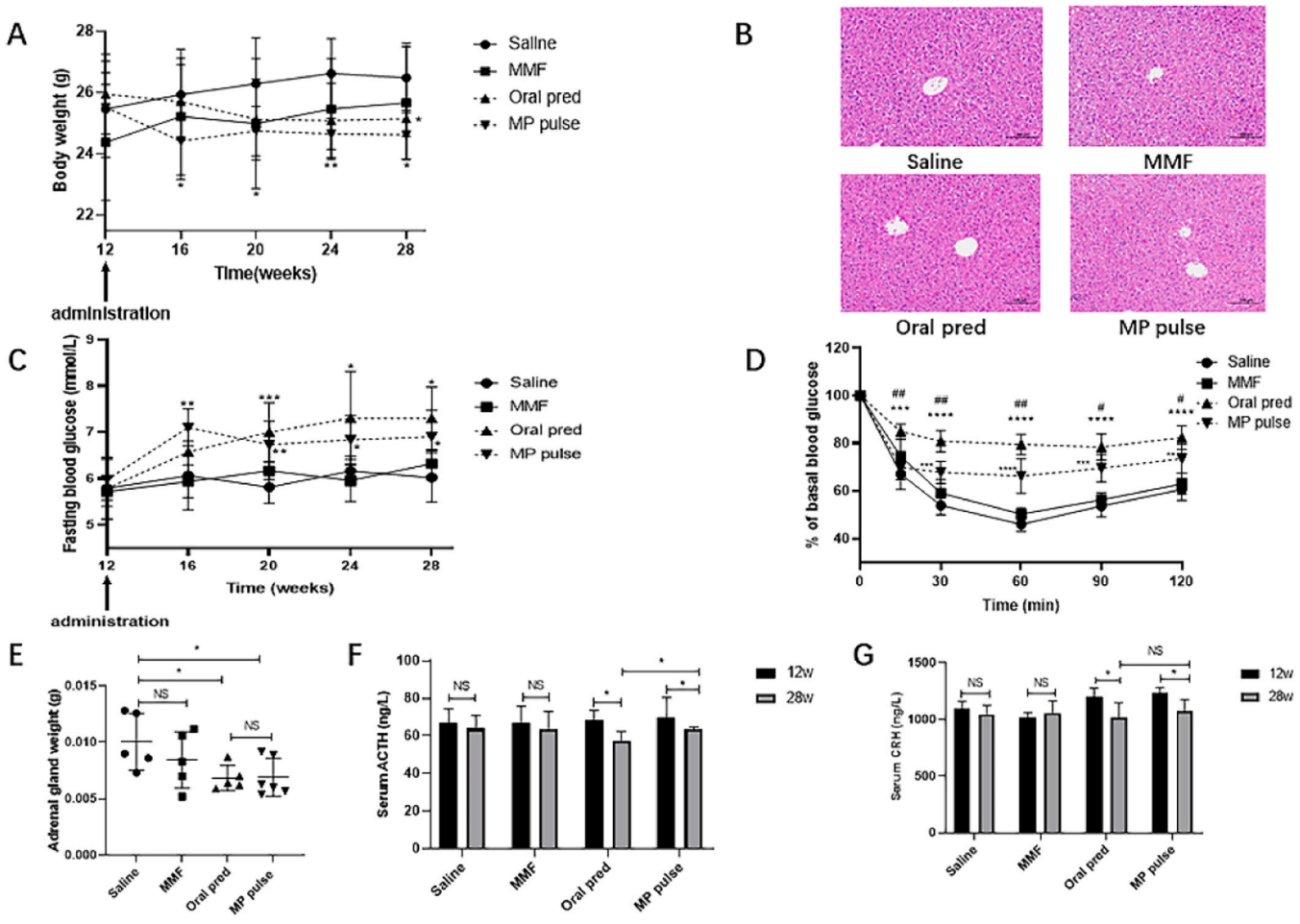

Fig. 4. Effect of different treatment groups on metabolic parameters. (A) Body weight of B6.MRL-Fas^{lpr}/J mice in each group. (* p<0.05, ** p<0.005 vs the saline group). (B) H&E-stained liver sections in different groups (saline, n=5; MMF, n=5; MP pulse, n=6; oral pred, n=5). Original magnifications×200. Scale bar=100μm. (C) Fasting blood glucose of B6.MRL-Fas^{lpr}/J mice in each group. (* p<0.05, ** p<0.005, *** p<0.001 vs the saline group). (D) Insulin tolerance test in B6.MRL-Fas^{lpr}/J mice treated with saline (n=5), MMF (n=5), MP pulse regimen (n=6) and oral pred regimen (n=5) at week 28. (* p<0.05, ** p<0.005, *** p<0.001, **** p<0.0001 vs the saline group; $^{\#}$ p<0.005, $^{\#\#}$ p<0.005, MP pulse group vs oral pred group). (E) Adrenal gland weight of mice surviving at the final time point (28 week), (saline, n=5; MMF, n=5; MP pulse, n=6; oral pred, n=5). Serum concentrations of ACTH (F) and CRH (G) at 12-week and 28-week time points (saline, n=5; MMF, n=4; MP pulse, n=5; oral pred, n=5). Results are expressed as mean ± SD. * p<0.05, ** p<0.005, *** p<0.001. NS represents no significant differences.

differ from that of mice in the saline group at week 20, but was significantly higher in the MP pulse group than in the saline group at 16 weeks (Fig. 4C). Notably, mice in the MP pulse group received the last MP pulse at week 17, after which the fasting blood glucose levels decreased substantially (at week 20) and stabilized. During the 16-week treatment period, no significant difference in fasting blood glucose level was observed between the MP pulse and oral pred groups (Fig. 4C). At the end of treatment (week 28), the ITT was performed in each group of mice. The saline and MMF groups exhibited similar responses (Fig. 4D). The MP pulse and oral pred groups showed insulin resistance, with the oral pred group showing significantly more resistance to insulin than the MP pulse group (Fig. 4D).

Chronic exposure to GCs suppresses the hypothalamic-pituitary-adrenal (HPA) axis. To clarify the inhibitory effects of different GC administration routes on the HPA axis, adrenal glands were harvested from mice in each group and weighed at necropsy. The adrenal glands were found to be atrophied in both the MP pulse and oral pred groups compared with those in the saline group (p=0.0342, p=0.0299, respectively; Fig. 4E). No significant difference in adrenal gland mass was observed between the MP pulse and oral pred groups (Fig. 4E). Furthermore, serum ACTH level decreased in the MP pulse and oral pred groups after 16 weeks of treatment (p=0.0373, p=0.0195, respectively; Fig. 4F). Serum CRH level also decreased after treatment with MP pulse and oral pred regimens (p=0.0248, p=0.0418, respectively; Fig. 4G). Notably, at 28 weeks, the oral pred group exhibited lower ACTH levels than the MP pulse group (p=0.0273; Fig. 4F), whereas no difference in CRH levels was observed between the two groups (Fig. 4G). These results indicate that compared with the oral pred regimen, the MP pulse regimen has lesser effect on glucose metabolism and the HPA axis.

3.4. Effects of MP pulse and oral prednisone regimens on bone quality and marrow adiposity in lupus-prone mice

Reduced bone mass and increased marrow adiposity are prominent side effects of GC treatment [33]. Therefore, we investigated the effects of the two GC regimens on bone quality and fat accumulation in the marrow. Lupus-prone mice exhibited sparse trabecular bone histology and severe fatty deposits in the bone marrow after GC treatment, which were more evident in the oral pred group (Fig. 5A). The trabecular BV/TV was 13.31 % and 16.05 % in oral pred group and MP pulse group; these values were significantly lower than in the saline group (25.09 %) (Fig. 5C). Similarly, the oral pred and MP pulse groups exhibited significantly reduced trabecular bone area and Tb. N versus the saline group (Fig. 5B and D). Interestingly, the above three parameters, which are closely associated with bone loss, were significantly more reduced in the oral pred group than in the MP pulse group (p=0.0162, p=0.039, p=0.049, respectively; Fig. 5B-D). Lower femur weight was also observed (p=0.0104, Fig. 5E). These results suggest that the MP pulse regimen has a lower impact on bone quality and trabecular structure and induces less bone marrow fat deposition.

Bone homeostasis is maintained by the coordination between bone formation and resorption [34]. Therefore, we explored the effects of the two GC regimens on bone resorption and formation. TRAP staining of femurs (Fig. 6A, upper panel) revealed that compared with the saline group, the oral pred and MP pulse groups showed a significantly increased area of osteoclasts in the trabecular bone (p=0.0008, p=0.0332, respectively; Fig. 6B). In addition, the osteoclast area was higher in the oral pred group than in the MP pulse group (p=0.0461, Fig. 6B). The expression of OCN, a bone formation marker, was detected

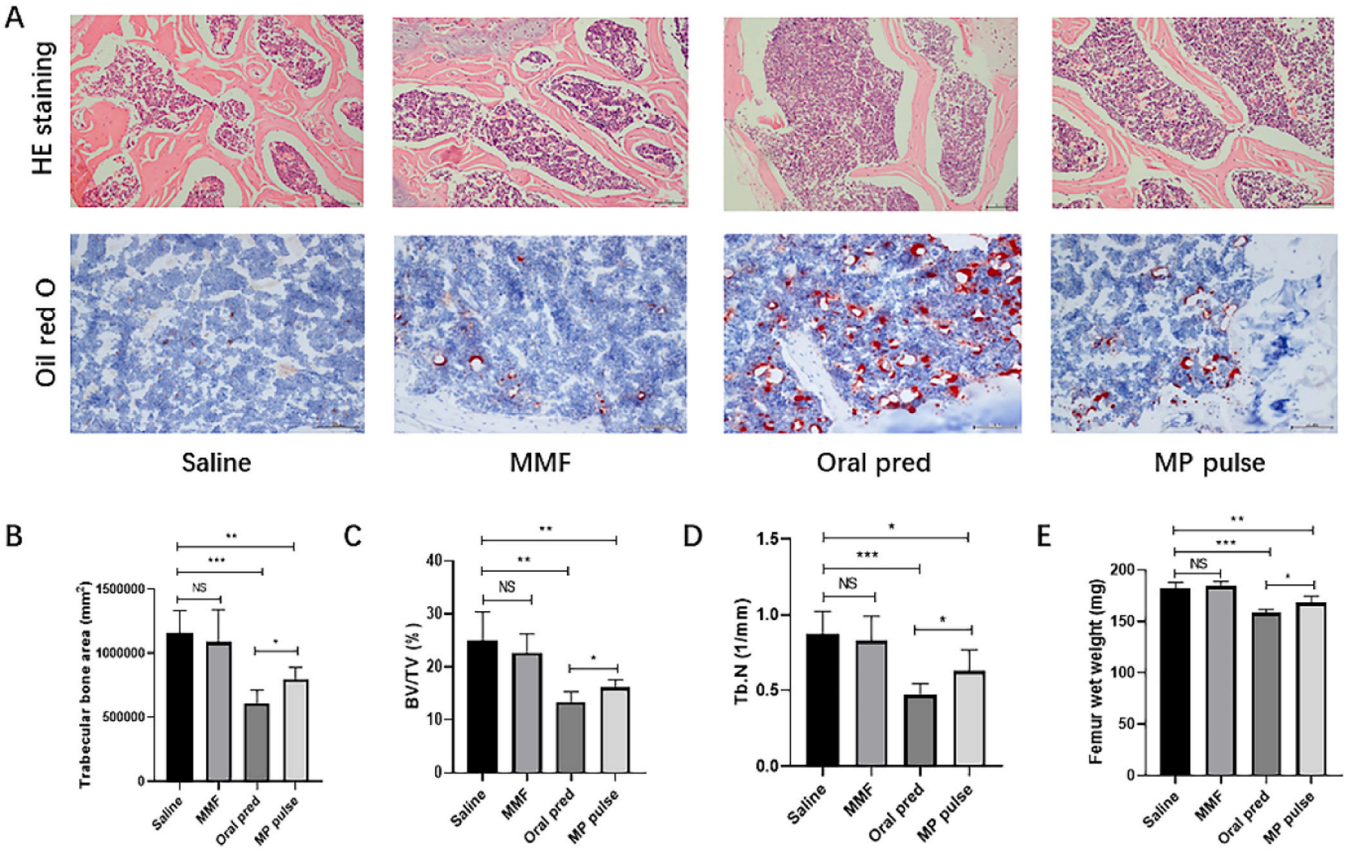

Fig. 5. Analyses of bone quality and marrow adiposity of different treatment groups. (A) Representative images of H&E staining of distal femurs (upper panel) and Oil Red O staining of bone marrow (lower panel) of B6.MRL-Fas^{lpr}/J mice in each group (n=5 per group). Original magnifications×200. Scale bar=50μm. (B-D) Quantitation of trabecular bone parameters, including trabecular bone area, BV/TV and Tb. N (n=5 per group). (E) The femur weight of B6.MRL-Fas^{lpr}/J mice in each group (n=5 per group). Results are expressed as mean ± SD. * p<0.05, ** p<0.005, *** p<0.001. NS represents no significant differences.

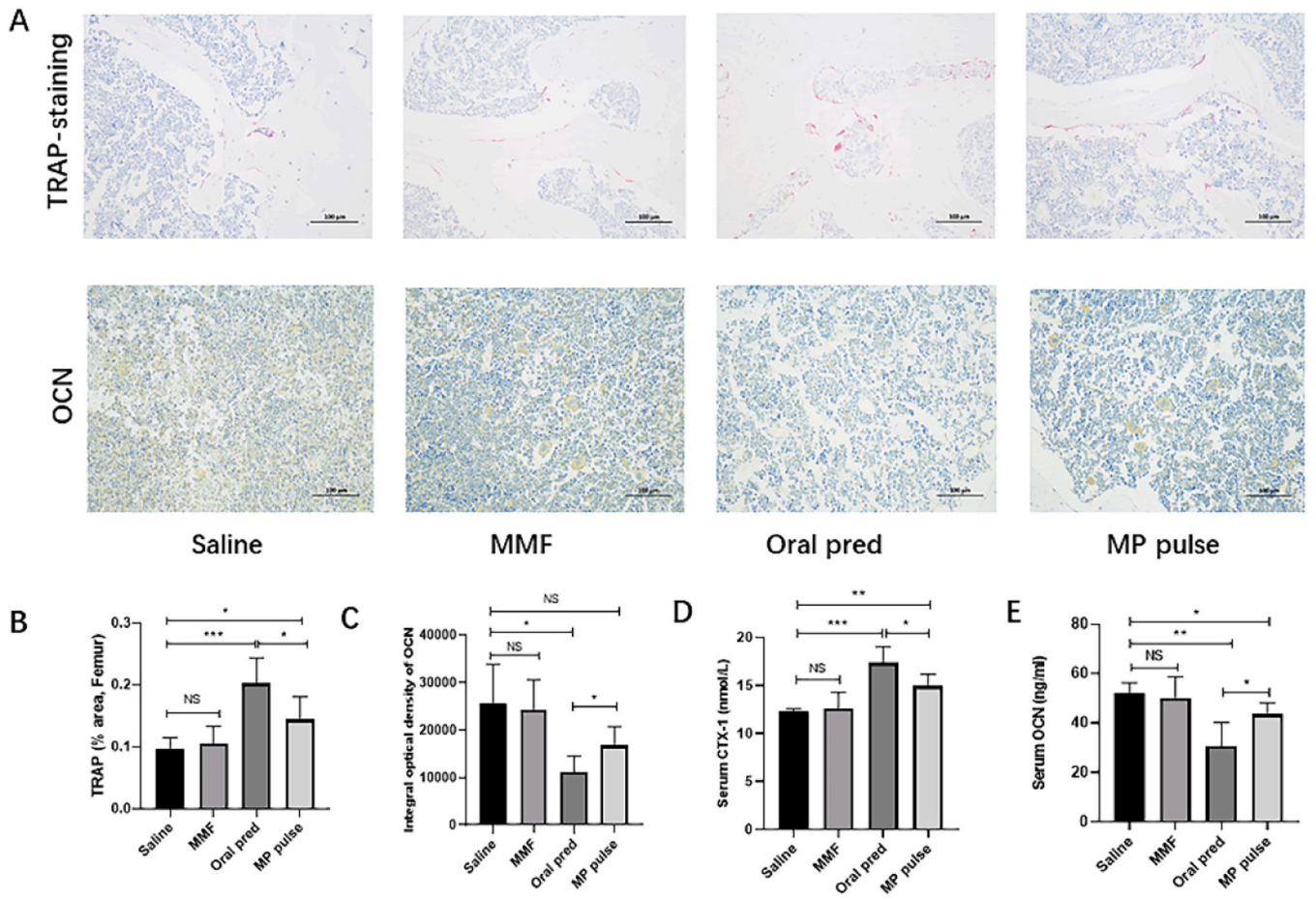

Fig. 6. Analyses of bone homeostasis of different treatment groups. (A) Representative TRAP-staining in the distal femur of trabecular (upper panel) and the expression of OCN in bone marrow detected by immunohistochemistry (lower panel) in each group (n=5 per group). Original magnifications×200. Scale bar=100μm. (B) Quantitation of TRAP positive cell in each group (n=5 per group). (C) IOD of OCN positive signals in each group (n=5 per group). (D) Serum levels of CTX-1 in B6.MRL-Fas^{lpr}/J mice after treated with different regimens (n=5 per group). (E) Serum levels of OCN in B6.MRL-Fas^{lpr}/J mice after treated with different regimens (n=5 per group). Results are expressed as mean ± SD. * $p<0.05$, ** $p<0.005$, *** $p<0.001$. NS represents no significant differences.

using immunohistochemistry. As shown in Fig. 6A (lower panel), OCN protein was expressed in bone tissue. We used IOD to assess the expression of OCN. Compared with the saline group, the oral pred group showed a decreased IOD of OCN (p=0.0006, Fig. 6C), and only a decreasing trend was observed in the MP pulse group (p=0.0585, Fig. 6C). In contrast to the TRAP staining results, OCN expression was lower in the oral pred group than in the MP pulse group (p=0.0435, Fig. 6C). Similarly, the two GC regimens significantly increased the concentration of CTX-1, a serum bone resorption marker (Fig. 6D) and decreased serum OCN levels (Fig. 6E). Compared with the MP pulse group, the oral pred group showed a significantly increased serum CTX-1 level (p=0.0014, Fig. 6D), although the OCN level decreased in this group (p=0.0262, Fig. 6E). These results suggest that the MP pulse regimen has a weaker effect on bone homeostasis, with less bone resorption and stronger bone formation, than the oral pred regimen.

3.5. The advantages of the MP pulse regimen may be associated with the overexpression of GILZ

To explore the mechanism responsible for the superior effects of MP pulse therapy over oral pred therapy, we first examined GRα mRNA expression using qPCR. GRα expression in the liver and bone was approximately 5-fold higher in the MP pulse and oral pred groups than in the saline and MMF groups (Fig. 7A and D). No difference in GRα mRNA expression was observed between the MP pulse and oral pred groups. Then, we examined the expression of the GC target gene GILZ, in the liver and bones. The expression of GILZ was upregulated by GCs, with the upregulation of GILZ expression being higher in the MP pulse group than in the oral pred group (p=0.0459, Fig. 7B). Similar results were obtained for GILZ expression in bone (p=0.0468, Fig. 7E). Considering GILZ downregulates PPARγ_2, a regulator of adipocyte differentiation, we further examined the expression levels of PPARγ_2 in liver and bone. Notably, PPARγ_2 expression was upregulated by the two GC regimens. The upregulation of PPARγ_2 expression was lower in the MP pulse group than in the oral pred group in bone (p=0.0319, Fig. 7C) but not in the liver (Fig. 7F). Based on these results, we hypothesized that the MP pulse regimen would have fewer side effects than the oral pred regimen, which may be associated with the overexpression of GILZ.

qPCR analysis of GRα (A), GILZ (B) and PPARγ_2 (C) in the liver of B6.MRL-Fas^{lpr}/J mice (n=5 per group). qPCR analysis of GR (D), GILZ (E) and PPARγ_2 (F) in the bone of B6.MRL-Fas^{lpr}/J mice (n=5 per group). Results are expressed as mean ± SD. * $p<0.05$, ** $p<0.005$, *** $p<0.001$. NS represents no significant differences.

4. Discussion

Despite the advancement in new therapies, including biologics, 68 % of patients with SLE still require GC therapy [35]. Considering the potent anti-inflammatory effects and irreplaceability of GCs in the clinical management of lupus and the toxicities associated with their chronic use [36], in this study, we sought to identify an optimal GC regimen to retain the anti-inflammatory effects of GCs whilst

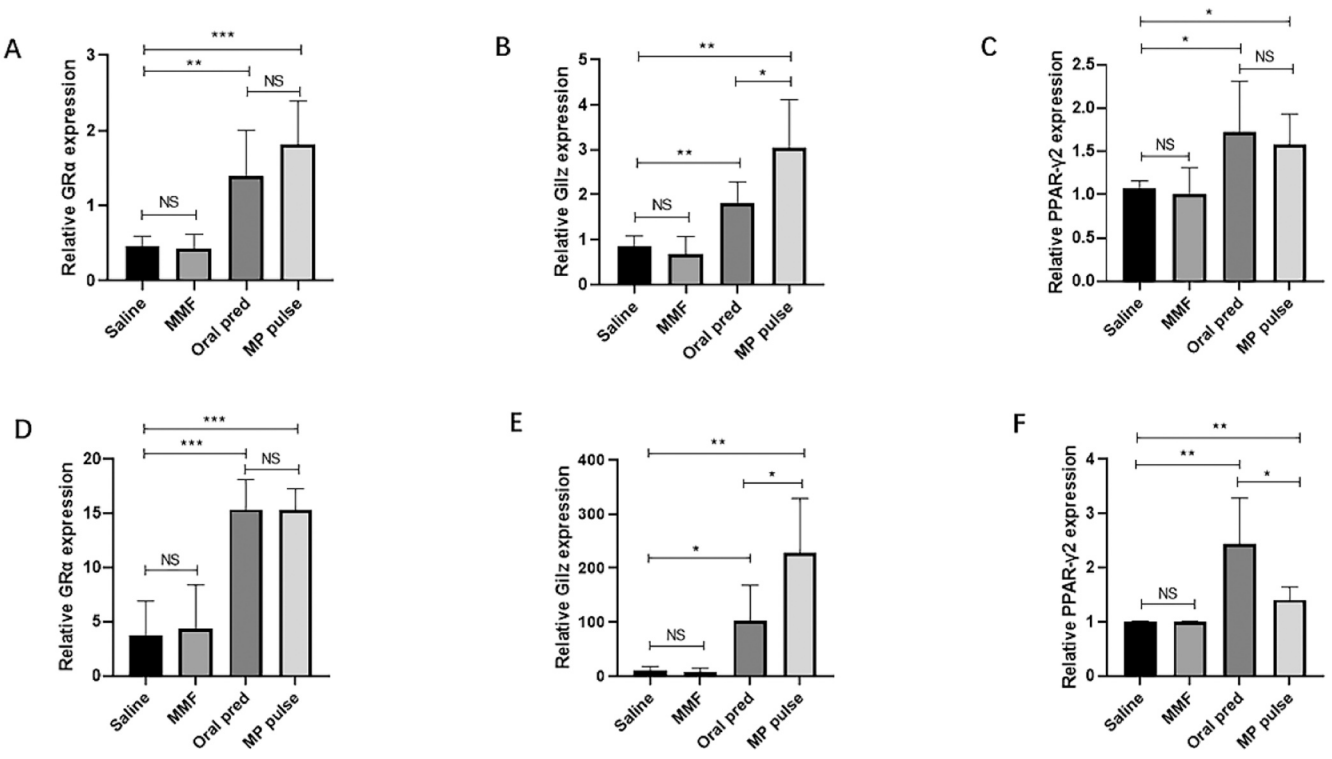

Fig. 7. The expression of GR and GCs response genes in B6.MRL-Fas^{lpr}/J mice.

minimizing side effects.

Type I interferons (IFN) play a central role in SLE pathogenesis [37]. Over 50 % of patients with SLE exhibit overexpression of type I IFN pathway genes in peripheral blood cells [38]. In addition, >75 % of GC-induced genes are regulated by IFN-α [39]. Guiducci et al. found that IFN-stimulated genes were not suppressed by oral GCs but were sensitive to MP pulse therapy, and the inhibition of IFN-signature recovered after eight days [40]. Recently, the Lupus-Cruces protocol has received more attention in the treatment of lupus nephritis (LN). This protocol can provide additional therapeutic benefits, which may be explained by the findings of Guiducci et al. The Lupus-Cruces protocol involves the administration of the MP pulse regimen combined with a rapidly tapered dose of oral prednisone. In class III and class IV LN, 6–9 fortnightly MP pulses were given, according to clinical response [14,41]. Therefore, we designed this MP pulse regimen and compared it with the conventional oral pred regimen in B6.MRL-Fas^{lpr}/J mice.

In the present study, similar to the oral pred regimen, the MP pulse regimen effectively reduced the serum anti-ds DNA and IgG levels and increased the serum C3 levels in B6.MRL-Fas^{lpr}/J mice (Fig. 2A-C). This finding was consistent with the immunosuppressive effects of GCs. B6.MRL-Fas^{lpr}/J mice exhibit a loss of function mutation in the death receptor Fas/CD95 gene. These lupus-prone mice develop massive lymphoproliferation and visceral-organ damage [42]. GCs induce lymphocyte apoptosis, which contributes to immunosuppression [43]. We found that, similar to the oral pred regimen, the MP pulse regimen reduced spleen index and lymph node scores (Fig. 2D, E). Furthermore, both of the two GC regimens improved the survival rate of B6.MRL-Fas^{lpr}/J mice throughout the experimental period, with no significant difference between the two regimens (Fig. 2F).

These results were further supported by the glomerular histological findings, in which the MP pulse and oral pred regimens showed similar therapeutic effects on glomerular injury, with disease severity rated mostly as mild to moderate (Fig. 3A, C). The efficacy of the two regimens in reducing urinary protein levels was also similar (Fig. 3B). All of these findings suggest that the two GC regimens have similar efficacy in treating SLE. This is different from our expectation, which was that the MP pulse regimen had a better efficacy. This unexpected result may be related to the comparable total GC dosages between the two regimens in this study. In fact, clinical studies have shown that higher doses of MP pulse therapy do not cause additional side effects [44]. We speculate that it would also be acceptable to apply higher doses of MP pulses in the clinic. In addition, the mice in our study lost weight after GC treatment (Fig. 4A). In contrast to the results obtained from long-term use of GCs in humans, GCs caused weight loss in mice, which was considered a side effect of the GCs [21]. Therefore, this result cannot be directly generalized to humans.

Exogenous GCs increase the risk of associated metabolic complications, including fat mass, fat distribution, and glucose metabolism. A previous study reported increased fat mass and reduced insulin sensitivity in NCD mice after 12 weeks of dexamethasone treatment [45]. However, in the present study, MP pulse and oral pred regimens did not induce lipid deposition (Fig. 4B). This may be explained by sexual dimorphism [46] and differential tissue responses [47] to chronic GC exposure. Intriguingly, although we found similar effects of the two GC regimens on fasting blood glucose levels in B6.MRL-Fas^{lpr}/J mice (Fig. 4C), insulin resistance was more pronounced in the oral pred group (Fig. 4D). This may reflect an intact response to GC-induced hyperinsulinemia [48]. These results indicate that disturbances in glucose homeostasis do not cause increased fat mass and that the MP pulse regimen is more advantageous than the oral pred regimen in stabilizing glucose metabolism.

As indicated by serum ATCH levels, the HPA axis in B6.MRL-Fas^{lpr}/J mice was less suppressed in the MP pulse group than in the oral pred group (Fig. 4E-G), possibly because of the negative feedback from GCs acting first on the pituitary gland [32]. A previous study suggested that less profound disturbances in the HPA axis may underlie metabolic disorders [49]. However, the published data supporting this idea are contradictory. Early reports posit that both osteoblasts and osteoclasts are major determinants in the control of glucose and lipid metabolism [24,50]. An imbalance between osteoblast-mediated bone formation

and osteoclast-mediated bone resorption contributes to the pathogenesis of osteoporosis. The present study revealed that the MP pulse regimen had fewer deleterious effects on bone mass and trabecular histology in the bone tissue of B6.MRL-Fas^{lpr}/J mice than the oral pred regimen (Fig. 5). Similarly, the MP pulse regimen induced less disruption of the balance between bone resorption and formation (Fig. 6). This is consistent with our results regarding glucose metabolism.

Therefore, we suggest that the efficacy of the MP pulse regimen in alleviating SLE symptoms in B6.MRL-Fas^{lpr}/J mice is similar to that of the oral pred regimen; however, the MP pulse regimen exhibits superior safety profile. This is not surprising given it is well known that GCs exert their anti-inflammatory effects via genomic and non-genomic mechanisms [51]. Non-genomic effects play an important role in mediating the therapeutic effects of GC pulse therapy [22]. Genomic effects are often the primary activation mechanisms of oral GC therapy. The toxicity and anti-inflammatory effects of the genomic effects increase in parallel with the dose. However, the activation of non-genomic effects provides additional benefits to patients with no increased toxicity [52]. Intravenous MP shows a rapid peak with a subsequent serum half-life of 3 hours [53]. In this MP pulse regimen, MP pulses were administered intermittently. The GC dose was high during the pulses; however, the metabolism was fast. The dose administered during the intermittent and maintenance periods was low, reducing the influence of GCs on the body in the treatment period [17]. However, these are all speculations, and the exact mechanism is not entirely clear.

In the present study, we further explore the mechanisms underlying the advantages of the MP pulse regimen. GCs act through GR, which is expressed in almost all cells in the body [54]. Insulin resistance and bone loss were milder in the MP pulse group; therefore, the liver and bone were the main tissues investigated in this study. We found that GR was not responsible for the reduction in side effects of the MP pulse regimen (Fig. 7A and D). GILZ is a GC response gene, whose expression is negatively correlated with SLE disease activity [55]. The results of the present study showed that upregulation of GILZ expression was significantly higher in the MP pulse group than in the oral pred group (Fig. 7B and E), suggesting the important role of this gene. GILZ increased osteoblast differentiation and inhibited adipocyte formation, which was attributed to the reduced expression of PPARγ_2 [56]. No difference in PPARγ_2 mRNA expression in the liver was observed between the MP pulse and the oral pred groups; however, a difference was observed in the bone. This is consistent with our finding that the MP pulse regimen induced less bone marrow adiposity and bone loss (Fig. 5). A recent study reported that overexpression of GILZ prevents IFN-stimulated gene upregulation in response to IFNα, negatively regulating the auto-amplification loop of the IFN response [57]. As mentioned earlier, IFN-stimulated genes are not suppressed by oral GCs but are sensitive to MP pulse therapy [40]. Therefore, we hypothesized that GILZ might reduce the GC-related side effects of MP pulses by modulating IFN-stimulated genes. Of note, in addition to GCs, interleukin (IL)-4, IL-10, and curcumin can also induce GILZ expression [58,59]. Therefore, the role of GILZ in this context warrants further study.

The present study had several limitations. First, GC-related bone destruction has been observed at various skeletal sites in the body, most notably in the lumbar spine [60]. In our study, exploration of the effects of GCs on bone was conducted on the femur. However, this did not significantly impact bone-related indicators because no matter which part of the bone tissue could partially reflect the effect of the two GC regimens on bone. Second, B6.MRL-Fas^{lpr}/J mice, as a murine model of lupus, are characterized by autoreactive lymphocyte accumulation and increased autoantibodies. This model emphasizes the importance of Fas-mediated peripheral tolerance in SLE pathogenesis [61]. It is well known that the pathogenesis of SLE is complex and the dysregulation of apoptosis is not the only mechanism involved in SLE development. Therefore, this murine model of SLE does not completely mimic human SLE, and our results must be interpreted with caution. Finally, the mechanism underlying the superiority of the MP pulse regimen over the oral pred regimen in treating SLE remains unclear. The exploration of GILZin this study is preliminary and not in-depth. Therefore, further studies are required.

5. Conclusion

Similar to the standard oral prednisone regimen, the MP pulse regimen produced a sustained therapeutic effect in female B6.MRL-Fas^{lpr}/J mice. However, the MP pulse regimen caused less insulin resistance, less HPA axis inhibition, as well as less bone loss and bone marrow fat deposition. The superior effects of the MP pulse regimen may be attributable to GILZ overexpression. The MP pulse regimen is a potentially promising GC treatment option for SLE.

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CRediT authorship contribution statement

Sirui Yang: Supervision, Funding acquisition. **Congcong Liu:** Methodology, Funding acquisition. **Jinxiang Liu:** Investigation. **Lishuang Guo:** Methodology. **Lu Pan:** Writing – review & editing, Writing – original draft, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval

All the experiments were approved by the Subcommittee on Research Animal Care of the First Hospital of Jilin University.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117138.

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