International Journal of Agriculture and Biosciences 2024 xx(x): xx-xx.
https://doi.org/10.47278/journal.ijab/2024.093

RESEARCH ARTICLE
eISSN: 2306-3599; pISSN: 2305-6622

Enhancing Rheumatoid Arthritis Treatment by Subcutaneous Methotrexate Injections and Anti-IL-2 Antibody Synthesis

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ABSTRACT

Rheumatoid arthritis (RA), an incapacitating autoimmune disorder marked by joint inflammation, cartilage degradation, and bone erosion, often results in disability. Diagnosis relies on detecting Rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), offering insights into disease progression before symptoms emerge. While Methotrexate (MTX) is a standard RA treatment, concerns over insufficient responses and gastrointestinal side effects prompt exploration of subcutaneous MTX injections. This method, established in the USA and Europe and recently approved in Japan, presents a potentially safer and more effective alternative. Additionally, research indicates that elevated Interleukin-2 (IL-2) levels correlate with reduced regulatory T-cell (Treg) levels, exacerbating RA progression. Mouse models demonstrate promise in slowing RA through IL-2 antibody-mediated inhibition, a concept validated in clinical studies involving RA patients. The investigation extends to the potential use of subcutaneous MTX injections as a preferable treatment modality in Pakistan, suggesting a comprehensive approach to managing RA.

Keywords: Interleukin 2, Clinical Studies, Rheumatoid Arthritis, Methotrexate, Antibodies

Article History
Article # 24-563a
Received: 04-Feb-24
Revised: 03-Mar-24
Accepted: 15-Mar-24

INTRODUCTION

Rheumatoid arthritis (RA) is a progressive autoimmune disease that primarily affects the body's joints, causing joint swelling, cartilage damage, bone erosion, and joint disability (Lee et al., 2012). Initially, only a few joints are impacted, but as the disease advances, it often involves most joints, accompanied by extra-articular symptoms. Genetic diversity, accounting for 50% of risk factors, plays a significant role in RA's multifactorial nature, with genetic markers like HLA-DR4 and HLA-DRB1 associated with the disease. Environmental factors, especially smoking, can trigger RA, particularly in those with a genetic predisposition, while infections do not directly cause RA (Pol et al., 2020).

RA is characterized by synovial cell proliferation known as Pannus, which leads to cartilage degradation and bone erosion (Cheng et al., 2024). The excessive production of pro-inflammatory cytokines like tumor necrosis factor (TNF) and interleukin-6 (IL-6) contributes to joint damage. RA risk increases with age and is higher in individuals with a family history of the disease. While RA is more common in females, the gender difference diminishes in older age (Wang et al., 2010).

Factors influencing RA risk include smoking, pregnancy, menarche age, menstrual regularity, breastfeeding, and the presence of specific antibodies such as rheumatoid factor (RF) and ACPA. The use of oral contraceptives or vitamin E doesn't significantly affect RA risk (Weismann et al., 1979). These antibodies are also

Cite this Article as: Sagheer S, Rasheed MS, Ashraf F, Rao A, Fatima M, Ajmal MN, Ameen A, Mohamed K, Khan A, Ali F, Ahmed A, Farzeen, Iqbal S, Arif T, Abid Z, Raza H, Mujeeb MT, Noel A, Batool R, Waqas M, Shahzadi K and Zafar MZ, 2024. Enhancing rheumatoid arthritis treatment by subcutaneous methotrexate injections and anti-IL-2 antibody synthesis. International Journal of Agriculture and Biosciences xx(x): xx-xx. https://doi.org/10.47278/journal.ijab/2024.093

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useful in diagnosing other autoimmune conditions. The presence of RF and ACPA is found in 50-80% of RA patients, and a positive antinuclear antibody test can predict juvenile RA prognosis. Elevated C-reactive protein (CRP) levels and erythrocyte sedimentation rate are associated with RA and used in tracking disease activity and treatment response (Salwen, 2011).

RA is a global condition, affecting people worldwide without regional or ethnic disparities (Ghoroghchian et al., 2020). The annual incidence is estimated at around 40/100,000 individuals globally, with a higher prevalence in women (2:1 to 3:1 compared to men). Both genetic factors, including HLA genes, and environmental factors like smoking contribute to RA risk. Early diagnosis and treatment are crucial, with DMARDs forming the cornerstone of RA treatment (Xia et al., 2008).

Methotrexate (MTX), a csDMARD, effectively manages RA by suppressing the overactive immune response. It's endorsed by leading medical organizations and well-tolerated,with hepatotoxicity, bone marrow toxicity, and gastrointestinal side effects being the primary concerns (Yoshida et al., 2001). The concurrent use of folic acid or folinic acid is recommended to counterbalance MTX's antifolate effects. Rheumatoid arthritis (RA) is a persistent autoimmune disease primarily affecting joints, characterized by inflammation, pain, stiffness, swelling, and progressive joint damage (Yüce et al., 2000). The treatment goal for RA is to reduce pain and inflammation, improving the patient's overall quality of life. One widely used treatment for RA is methotrexate (MTX), an antifolate agent that competitively inhibits dihydrofolate reductase, thereby suppressing the overactive immune response seen in RA patients (Zhao et al., 1995). MTX is classified as a conventional synthetic disease-modifying antirheumatic drug (csDMARD) known for reducing inflammation, controlling symptoms, preventing joint damage, and potentially increasing patient survival (Bergmann et al., 2020; Ait-Oufella et al., 2021; Bonacina et al., 2021).

MTX has been a cornerstone of global RA treatment since the 1980s, praised for its effectiveness, affordability, and favorable safety profile (Cabello-Olmo et al., 2019; Cano-Martínez et al., 2020). Notably, the European Alliance of Associations for Rheumatology and the American College of Rheumatology (ACR) recommend MTX as a first-line treatment (Cano-Martínez et al., 2020; Chanouzas et al., 2019). The ACR even advocates MTX monotherapy over other DMARDs, biological DMARDs (bDMARDs), or targeted synthetic DMARDs (tsDMARDs). MTX monotherapy is preferred over dual or triple csDMARD therapy and combination therapy with MTX plus a tumor necrosis factor inhibitor, according to ACR guidelines (Deroissart et al., 2021). In Japan, oral MTX gained approval in 1999 for RA treatment in adults and is now considered the primary choice by the Japan College of Rheumatology (Dubey et al., 1993).

Initially, the maximum weekly dose of oral MTX in Japan was limited to 8mg, but in 2011, the Pharmaceuticals and Medical Devices Agency of Japan approved an expanded maximum dose of 16mg per week (Ferreira et al., 2021). However, even with this increase, Japanese doses of 6–8mg per week as a baseline and an escalated dose of 16mg per week remain lower than recommended doses in Western countries. This difference may be attributed to over half of Japanese patients experiencing intolerance to oral MTX doses of 16mg per week, possibly due to genetic polymorphisms affecting MTX metabolism (Karakus et al., 2020).

MTX is generally well tolerated, with its primary adverse drug reactions encompassing hepatotoxicity, bone marrow toxicity, and gastrointestinal (GI) side effects. To counteract the antifolate properties of MTX, the concurrent use of folic acid or folinic acid is advised, a practice endorsed by major clinical guidelines (Cano-Martínez et al., 2020; Deroissart et al., 2021; Dubey et al., 1993; Lee et al., 2012). While hepatotoxicity and bone marrow toxicity can often be managed by dose reduction or temporary discontinuation of the drug, managing nausea and other GI side effects is more challenging.

MATERIALS & METHODS

The study was designed by keeping all the inclusion and exclusion criteria set by Helsinki institute for the patients and animals used in this study. An informed consent was also taken from human patients before injecting them with MTX, patients' disease history was also carefully checked and monitored. During this study animals were carefully used by strictly following the guidelines of ARRIVE and after the approval of ethical committee of UMT (approval number 178/22) and Superior University, All the experimental protocols were approved by the institute and the experiment is being carried out. During the work no animal was sacrificed before injection of IL-2 antibody. While all the experimental protocols were approved, and consents were obtained from the patients during collection of this data at the hospital. Relevant data was collected from total RA patients (n= 105) for this study with 157 control (n= 157), 90 patients for MTX study by using demographics and disease activity measurements from Shalamar Hospital Lahore, Pakistan were selected and bring to Azra Naheed hospital for injection by keeping inclusion and exclusion criteria for RA patients. Injections and subcutaneous MTX Initial dose of local pharmaceutical industry was purchase from Lahore to treat patients of RA using oral MTX. Patients received their prescribed MTX treatment orally or subcutaneously, and the appropriate dosages were determined based on clinical guidelines. Subjects were monitored during the administration process. Blood samples were collected at specified time intervals post-administration to analyze MTX concentrations. Data was then processed to evaluate pharmacokinetic parameters. Statistical analysis involved comparing the pharmacokinetic data between oral and subcutaneous MTX groups. Descriptive statistics, such as mean values and standard deviations, were used to summarize the data. Relevant statistical tests were employed to determine significant differences.

Synthesis of Mouse Anti-Rheumatoid Antibody

The conjugation of bovine serum albumin (BSA) with IL-2 antibody, as well as ovalbumin with IL-2, was carried

out using a two-step glutaraldehyde method. To activate BSA, 200 mg of it was dissolved in a 10mL solution of pH 6.8 sodium phosphate buffer, and 13mL of 5% glutaraldehyde was added. Following dialysis in pH 6.8 phosphate-buffered saline (PBS), BSA was conjugated with the anti-RA interleukin medication. This conjugation involved dissolving the medication in 2mL of the activated BSA solution and cross-linking the mixture with 50µL of 200mM glutaraldehyde. Subsequent dialysis was carried out in pH 7.4 PBS, resulting in the final conjugate, which was stored at 4°C. Similarly, ovalbumin was activated by dissolving 200 mg in a 10mL pH 6.8 sodium phosphate buffers and adding 13mL of 5% glutaraldehyde. After dialysis in pH 6.8 PBS, the activated ovalbumin was mixed with a solution of 10mg/mL anti-RA IL-2 in 0.5M pH 9.7 sodium carbonate buffer. The resultant mixture was then combined with 2mL of the activated ovalbumin serum and cross-linked using 50µL of 200mM glutaraldehyde at 23°C. The final conjugate was obtained (Table 1) following dialysis in pH 7.4 PBS and was stored at 4°C (Zhao et al., 2005).

For the subsequent in vivo studies, mice were injected with the BSA-conjugated medication. Intravenous injections, administered once every seven days, were used to evaluate the generation of anti-tuberculosis (TB) antibodies (Zimmermann et al., 2019).

Table 1: Immunization and injection formulation on different days

Date	Day	Injection formulation	
		Drug (µL)	Saline (µL)
09-08-2022	Monday	200	800
16-08-2022	Monday	300	700
24-08-2022	Tuesday	400	600
30-08-2022	Monday	500	500
07-09-2022	Tuesday	500	500

Saline = 1% NaCl.

Subcutaneous immunization was performed by injecting the antigens intravenously into the mice along with adjuvant, maintaining a 1:1 ratio between adjuvant and antigen. The injection was delivered into the subcutaneous layer located beneath the skin's epidermal layer. This method allowed for the gradual release of the antigen into the host. Subcutaneous immunization required a one-week interval between injections to prevent potential resistance to the antigen due to over-vaccination (Riggio et al., 1982).

Immunization

Once the mouse had been immunized as shown in Table 1, the plasma was taken in order to do further procedures including ELISA, western blotting, and dot blotting to check for the existence of an anti-TB antibody.

To confirm the presence of mouse anti-RA antibodies, a cardiac puncture procedure was conducted on a mouse following a 4-week subcutaneous vaccination. This allowed for the collection of blood, which was then allowed to clot. The resulting serum was carefully separated and stored at -20°C for subsequent testing. The presence of anti-RA antibodies was verified through two different assays (Rocchi et al., 1994).

In the first assay, an immune dot blot analysis was employed. A nitrocellulose membrane was loaded with two sets of substances: one with bovine serum albumin (BSA) as a control and the other with BSA conjugated with the drug as the test, both suspended in PBS. The membrane was blocked to prevent nonspecific binding, washed, and then incubated with a diluted mouse serum solution. After a defined incubation period, it was washed again and treated with a secondary antibody. The reaction was completed by adding a substrate solution to assess color changes, confirming the presence of the antibodies (Russell et al., 1983).

The second assay, an enzyme-linked immunosorbent assay (ELISA), was conducted by using microtiter plate wells. One well contained ovalbumin serum conjugated with the drug, and the other contained bovine serum albumin conjugated with Interleukin. To block non-specific binding, a solution of 5% Tris-buffered saline skim milk was added to each well. Mouse serum was applied to the test wells, followed by incubation. After washing, an enzyme linked to the secondary antibody was introduced, and the plate was incubated once more. Color changes in the wells were monitored using a substrate solution, indicating the presence of the antibodies. These procedures collectively ensured the accurate detection of mouse anti-RA antibodies (Sandstead, 2000).

Data Analysis

Using SPSS 2.0, data were analyzed by finding probability between control and experimental group for MTX injection.

RESULTS AND DICSUSSION

Subcutaneous Immunization in Mice

The method for subcutaneous immunization of mice with BSA-activated drug entails the injection of mice with a drug chemically linked or conjugated to Bovine Serum Albumin (BSA). This process is designed to elicit an immune response in the mice, resulting in the production of antibodies directed against the drug-BSA complex. Subcutaneous immunization, achieved through the injection of the complex beneath the skin, is a widely employed approach for inducing a targeted immune response. This method is utilized to generate antibodies capable of recognizing and potentially neutralizing the drug. Such antibodies are valuable in various research and therapeutic contexts, especially when the drug serves as an antigen to stimulate antibody production.

Immuno Dot Blot of Mouse Serum

The negative result of the dot blot signifies that specific antibodies against BSA were not detected in the mouse serum. This suggests that BSA did not induce an immune response in the mouse, and no antibodies targeting BSA were present in the serum. In this experiment, we conducted a dot blot assay to assess the immune response of mouse serum to BSA and IL-2. The objective was to determine whether mouse serum contains specific antibodies targeting these antigens.

1. **BSA Dot Blot**

The dot blot assay with BSA as the antigen revealed a positive result. There were distinct spots or dots on the membrane, indicating the presence of antibodies in the mouse serum that recognize and bind to BSA. This suggests an immune response to BSA in the mouse serum.

2. **IL-2 Dot Blot**

Similarly, the dot blot assay with IL-2 as the antigen also produced a positive result. Clear spots or dots were observed on the membrane, demonstrating the presence of antibodies in the mouse serum that specifically bind to IL-2. This suggests an immune response to IL-2 in the mouse serum.

The positive immune dot blot results for both BSA and IL-2 indicate that the mouse serum contains specific antibodies directed against these antigens. This immune response may have important implications for understanding the immune system's recognition and response to these particular molecules.

These findings provide valuable insights into the immunogenicity of BSA and IL-2 in the context of mouse serum and may be relevant in various research or clinical applications. Further studies can be conducted to explore the specificity, quantity, and potential applications of these antibodies in greater detail.

- **ELISA to detect anti–Interleukin-2 Antibodies**

In contrast, the control samples showed negative results in the ELISA. This suggests that there were no detectable anti-IL-2 antibodies in the control samples. The optical density readings for the control samples remained at background levels, confirming the absence of specific antibodies targeting IL-2.

These results indicate that there is a significant presence of anti- IL-2 antibodies in the test samples, while the control samples do not exhibit this immune response. These findings have important implications for understanding the immune response to IL-2 and may be relevant to various clinical or research contexts where IL-2 is a target of interest. Further investigations and experiments may be needed to explore the implications and applications of these results in greater detail.

Synthesis of Magnetic Nanoparticles (MNPs)

The MNPs are synthesized using a co-precipitation method, which involves the combination of $FeCl_2$ and $FeCl_3$ in a basic solution to form Fe_3O_4 nanoparticles. Initially, 8.110g of $FeCl_3$ and 3.168g of $FeCl_2$ are weighed. Subsequently, 50mL of $FeCl_2$ solution and 100mL of $FeCl_3$ solution are prepared. The turbid $FeCl_2$ solution is placed on a magnetic stirrer and left overnight after which it is filtered. The solutions of $FeCl_2$ and $FeCl_3$ are then combined. The iron solution's pH is adjusted to 10 by adding NH_4OH solution while maintaining the mixture at 80°C on a magnetic stirrer. The solution is heated for 45min. Upon reaching a pH of 7 (requiring 20-25 washings), it is thoroughly rinsed with warm water, involving 30ml of water and 70mL of ethanol in the first addition. The MNPs are separated by applying a magnetic field, and these particles are subsequently placed in an oven set to 40°C for drying.

Conjugation of MNPs with Anti-interleukin Antibodies

Polyclonal anti-interleukin antibodies were conjugated to MNPs through the carbodiimide method, facilitating the binding of the NH group of the MNPs with the carboxyl group of the antibodies. This conjugation strategy preserves the antigen-binding site on the antibodies, allowing them to selectively bind interleukin. These functionalized MNPs can be precisely directed to the affected joint site utilizing a magnetic field. This targeted delivery approach is especially relevant in cases of RA where cytokine overproduction triggers localized inflammation.

Characterization of MNPs

Characterization is a crucial step undertaken to assess the synthesis, properties, and composition of MNPs. The characterization of MNPs involved a comprehensive analysis through microscopic examination and spectrophotometric techniques.

Microscopic Analysis

The microscopic images of MNPs, which have been conjugated with polyclonal anti-interleukin antibodies and stained, clearly reveal the presence and distribution of nanoparticles within the sample. These images provide visual evidence of the successful conjugation process and the specific binding of antibodies to the nanoparticles. The stained area in the images highlights the localization of nanoparticles, confirming their presence in the sample. This characterization is essential for verifying the effectiveness of the conjugation process and ensuring the nanoparticles are suitable for their intended applications, such as targeted drug delivery or diagnostics shows in Fig. 1.

Spectrophotometric Analysis of MNPs

Fig. 2 shows the spectrophotometric analysis of MNPs yielding noteworthy findings. Specifically, the analysis revealed a shift in the absorption peak from 410 to 430 units of absorbance when the nanoparticles were bound to polyclonal anti-interleukin antibodies.

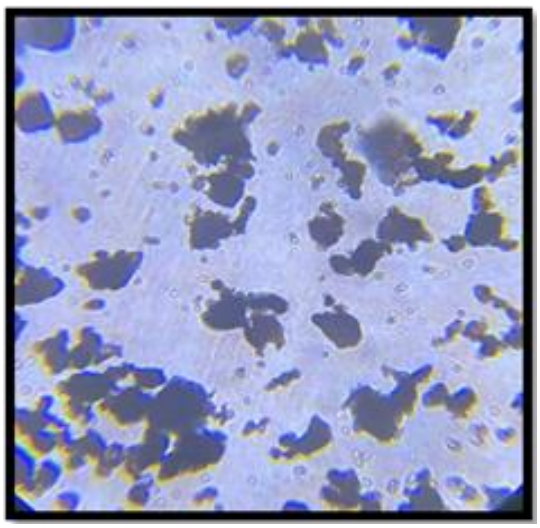

Fig. 1: Microscopic examination of magnetic nanoparticles conjugated with anti-interleukin antibodies.

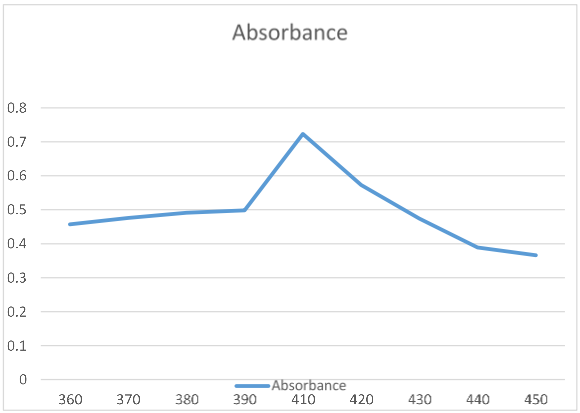

Fig. 2: Representation of absorbance before antibody binding at different wavelength.

Fig. 3 shows the shift in the absorption peak is a significant indicator of the successful conjugation of antibodies to the nanoparticles. The change in the absorption peak is attributed to alterations in the nanoparticles' electronic or chemical environment resulting from their interaction with the antibodies and after absorption with attached antibodies. This indicates that the antibodies have indeed attached to the nanoparticles, potentially occupying specific binding sites on the particle surface. Such a shift in the absorption spectrum is a valuable confirmation of the conjugation process and provides essential insights into the functionalization of these nanoparticles, which is pertinent for their intended applications, such as targeted drug delivery or immunoassays.

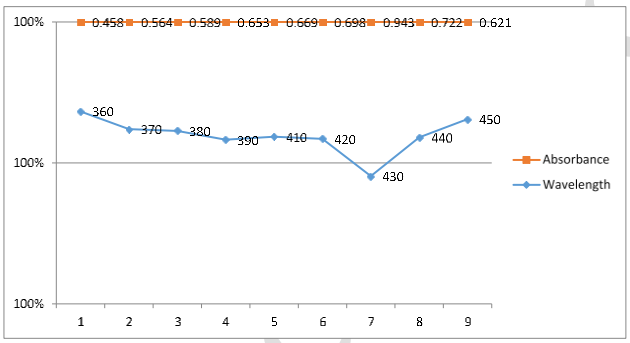

Fig. 3: Illustration of high absorbance 0.9 at 430nm wavelengths after binding of antibody.

Mechanism of ELISA to Detect Anti-IL-2 Antibodies

ELISA for anti- IL-2 antibodies works by immobilizing IL-2 on a plate. Specific anti-IL-2 antibodies in a sample bind to it. After washing, an enzyme-linked secondary antibody is added, and a substrate produces a signal if anti-IL-2 antibodies are present. The signal's intensity indicates antibody levels, enabling their detection. This method is crucial for research and diagnostics.

Fig. 4 represents IL-2 effects on patients while, Total RA patients (n=105) were selected for this study with 157 control from Shalamar hospital Lahore Pakistan. Table 2 presents data on the prevalence of specific antibodies, Anti-Interleukin 2 IgG and Anti-Interleukin IgA, in the context of RA and a control group. In RA patients, 26.0% exhibited Anti-IL-2 IgG, while only 6.8% of the control group tested positive. Similarly, 21.2% of RA patients had Anti-Interleukin IgA, compared to 5.3% in the control group. Sensitivity measures the ability of these antibodies to correctly identify RA patients, with values of 26.0% for IgG and 22.2% for IgA. Specificity assesses the ability to correctly exclude non-RA individuals, with values of 96.2% for IgG and 95.8% for IgA. Furthermore, the odds ratios (OR) for RA, which indicate the likelihood of having these antibodies in RA patients compared to controls, are 5.6 for IgG and 4.99 for IgA. These findings contribute valuable insights into the potential diagnostic significance of these antibodies in the context of RA.

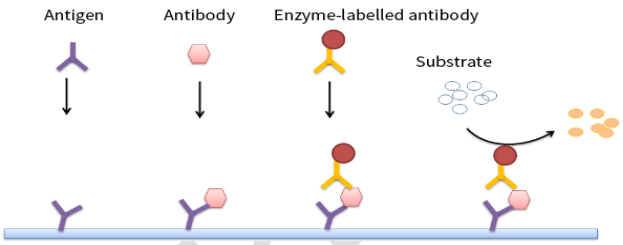

Fig. 4: Anti-IL-2 mouse anti body effect on patients.

Table 2 shows the comparison between RA patients and controls, significant differences were found in Anti- IL-2 IgG levels (P< 0.0005) and highly significant differences in Anti-IL-2 IgA levels (P<0.0001). No significant difference was observed in ACPA levels (P<0.7673) between these groups.

When comparing RA patients with erosive disease to those without erosions, significant differences were noted in both Anti-Interleukin-2 IgG (P<0.0156) and Anti-Interleukin-2 IgA levels (P<0.0166). However, there was no significant difference in ACPA levels (P<0.7673) between these subgroups. Additionally, among RA patients, those under biological treatment exhibited highly significant differences in Anti- IL-2 IgG levels (P<0.0003) and significant differences in Anti- IL-2 IgA levels (P<0.0008) compared to patients not on biologics. ACPA levels showed no significant difference in this comparison (P<0.7651) shown in Table 4 and its comparison in illustrated in Fig. 6. These findings underscore the varying antibody levels in different RA patient groups, emphasizing the potential clinical implications of these differences.

Table 3 provides initial methotrexate dosages based on the patient's weekly oral methotrexate prescription. It ensures a tailored transition, with initial doses of 8.5mg for 7-8mg oral MTX, 8.5-11mg for 9-10mg oral MTX, and 11-13.5 mg for 13-16 mg oral MTX, optimizing therapeutic precision. MTX Oral methotrexate (MTX) displays reduced bioavailability, particularly at doses exceeding 15mg (Roy et al., 2020). Studies revealed that the mean bioavailability ratio for MTX, considering a median weekly dose of 30mg (range 25–40mg), between oral and subcutaneous administration was 0.67 (range 0.24–0.97), favoring subcutaneous MTX (Shah and Mayor, 2022). Oral MTX's bioavailability plateaus beyond 15mg, while subcutaneous MTX exhibits a linear, dose-dependent increase (Mulholland et al., 2021). Subcutaneous MTX injections

result in higher exposure than equivalent oral doses, even at 24mg per week. The area under the plasma drug concentration-time curve (AUC0–t) consistently shows higher values after subcutaneous MTX administration across all dose groups assessed (Orrù et al., 2021). The AUC0–t ratios compared to reference were 137% after 8.5mg, 148% after 15mg, 153% after 23.5mg, and 169% after 30mg of MTX (Orrù et al., 2021). These findings imply limitations in the oral MTX absorption process through active transport. Moreover, potential partial degradation of oral MTX by the gut microbiome may contribute to differences in MTX availability compared to parenteral administration (Teh et al., 2021). Subcutaneous MTX effectively overcomes these limitations, ensuring enhanced and reliable bioavailability compared to oral MTX.

Table 2: Types of antibodies in rheumatoid arthritis patients and their prevalence in experimental and control groups

Antibody types	RA patients (n=105)	Controls (n=157)
Anti-interleukin 2- IgG (%)	26.0% (27/105)	6.8% (10/157)
Anti-Interleukin IgA (%)	21.2% (23/105)	5.3% (9/157)
Double Positivity (%)	14.5% (15/105)	2 (Controls)
Sensitivity (IgG)	26.0%	-
Specificity (IgG)	96.2%	-
Sensitivity (IgA)	22.2%	-
Specificity (IgA)	95.8%	-
Odds Ratio (OR) (IgG)	5.6 (RA)	-
Odds Ratio (OR) (IgA)	4.99 (RA)	-

Table 3*:* Shows dosage per week of shifting subjects to oral on subcutaneous

Oral Methotrexate (MTX) Dosage mg/week	MTX Initial dose (mg)
7-8	8.5
9 or 10	8.5-11
13-16	11 or 13.5

Fig. 5 shows MTX plasma concentration in the patients with the passage of time while Fig. 6 shows these parameters offer valuable insights into how the drug behaves within the body. The first parameter, AUC0–inf (Area Under the Curve from 0 to Infinity), measures the total exposure of the body to MTX, quantifying the drug's concentration in the bloodstream over time. In this case, it has a value of 1640±456ng h/mL. Cmax (Maximum Concentration) signifies the peak concentration of MTX in the bloodstream after dosing, with a value of 479±107ng/mL. It reflects the drug's highest level shortly after administration. Clearance (CL) is the rate at which the body eliminates MTX from the bloodstream. Here, the CL value is 6.46±1.56L/h, indicating how quickly the drug is removed from the body.

Volume of Distribution (Vd) represents the hypothetical volume into which the drug is evenly distributed at the same concentration as in the bloodstream. The Vd value for MTX is 26.0±4.78L. The Half-life (T1/2) measures the time it takes for half of the drug in the bloodstream to be eliminated. In this case, the half-life is 2.96±1.14 hours, indicating relatively quick clearance. Tmax (Time to Maximum Concentration) reveals the median time taken to reach the maximum drug concentration in the bloodstream. It occurs at 0.50 hours, with a range of 0.25 to 0.75 hours. This parameter highlights how rapidly MTX reaches its peak concentration after administration. These parameters collectively provide a comprehensive understanding of MTX's pharmacokinetics, assisting in assessing its efficacy, safety, and dosing strategies when utilized as a medication.

Higher concentrations of long-chain MTX polyglutamates have been linked to reduced joint swelling, lower disease activity, and improved MTX response in patients (Wu et al., 2020). Transitioning from oral to subcutaneous MTX elevates long-chain MTX Glu levels more rapidly, reaching 90% of steady-state levels quicker than oral MTX, potentially explaining the faster response to subcutaneous MTX. This is due to increased polyglutamation, leading to enhanced cellular retention, drug accumulation, and prolonged drug availability (Zhang et al., 2022).

The shift from oral to subcutaneous MTX results in an increase in long-chain MTX Glu, which has been consistently associated with reduced disease activity, indicating a potential superiority of subcutaneous MTX over oral administration (Wu et al., 2020; Zhang et al., 2022).

Fig. 7 provides a comprehensive comparison of adverse gastrointestinal (GI) effects observed in studies conducted on mice. It presents percentages of occurrence for two different administration routes: oral and subcutaneous (SC). In terms of overall GI disorders, the data indicates that the oral route results in a notably higher incidence at 35.0% compared to the subcutaneous route, which shows a significantly lower rate of 16.4%. When examining specific GI issues, it becomes apparent that nausea is considerably less prevalent with subcutaneous administration, standing at 4.8%, in contrast to oral administration, where it's notably higher at 15.0%. Furthermore, there's a distinction within the oral group, with two different oral administration methods (Oral1 and Oral2/3) showing varying percentages of 13.2 and 15.6%, respectively, while the SC route remains consistently lower.

Stomatitis, another GI concern, is slightly more common in the SC group at 6.8%, compared to the oral route at 5.0%. Interestingly, the oral method Oral2/3 presents a lower percentage of 2.1% compared to the other oral route (Oral1), which stands at 4.7%.

Constipation displays a higher occurrence in the oral group (7.0%) compared to the subcutaneous route, where it's notably lower at 2.9%. Lastly, diarrhea exhibits a

Table 4: Comparison of rheumatoid arthritis patients with control group on erosive disease and biological treatments

Parameters	RA Patients vs. Controls (P-value)	Patients with Erosive Disease vs. Without Erosions (P-values)	Patients Under Biological Treatment vs. Not on Biologics (P-values)
Anti-Interleukin-2 IgG	P=0.0005	P=0.0156	P=0.0003
Anti-Interleukin-2 IgA	P<0.0001	P=0.0166	P=0.0008
ACPA	-	P=0.7673	P=0.7651

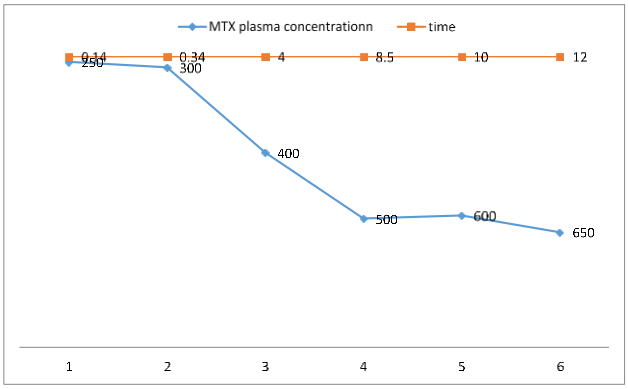

Fig. 5: Shows MTX plasma concentration on changes with time in patients.

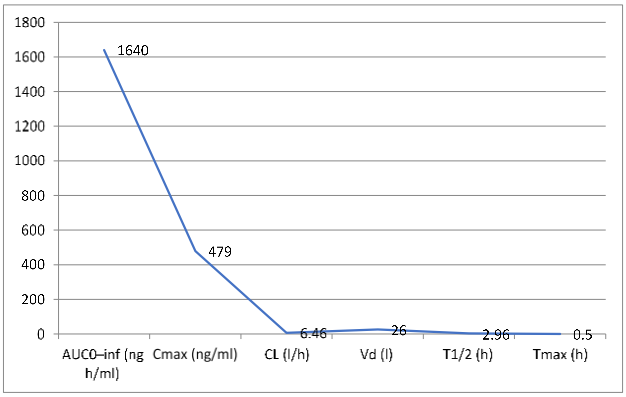

Fig. 6: Showing the pharmacokinetics of patients with locally manufactured MTX dose.

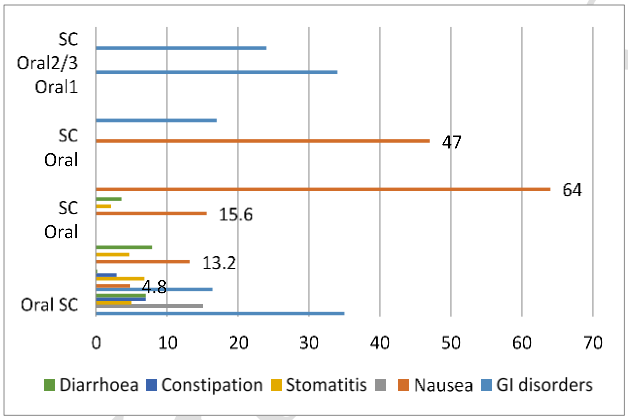

Fig. 7: Subcutaneous (SC) MTX once per week; Oral$_1$: single dose per week; Oral2/3: two or three doses per week.

significant difference, with the subcutaneous route reporting only 0.2%, contrasting with the oral method at 7.0%. Within the oral category, Oral2/3 stands out with a higher rate of 7.9% in comparison to Oral1, which presents a lower incidence of 3.6%.

In conclusion, this data demonstrates that subcutaneous administration appears to be associated with a reduced incidence of adverse GI effects, particularly in the context of nausea and diarrhea, compared to oral administration. These findings suggest potential advantages for subcutaneous routes in minimizing these specific side effects (Sasidharan et al., 2011).

Prior to a trial in Pakistani patients, a multicenter study compared subcutaneous to oral MTX in MTX-naïve patients with active RA. Subcutaneous MTX demonstrated superiority over oral administration after 24 weeks based on ACR20 and ACR70 responses (Scholmerich et al., 1986). The incidence of gastrointestinal adverse events was similar, but diarrhea was reduced in the subcutaneous group. Another study compared subcutaneous and oral MTX, with subcutaneous MTX showing higher efficacy and fewer adverse effects (Schölmerich et al., 1983). A split-dose regimen and once-weekly oral MTX were compared to intramuscular MTX, and no significant differences in adverse events were observed (Sengupta et al., 2015). However, more subjects discontinued the trial in the intramuscular and once-weekly oral groups due to non-compliance and discomfort with the method of application. In a 52-week extension trial, subcutaneous MTX with dose escalation based on disease activity and tolerability resulted in clinical improvement, with over half of participants achieving remission or low disease activity (Sirelkhatim et al., 2015).

Rheumatoid arthritis is a complex autoimmune disease characterized by chronic inflammation, the precise origins of which remain unclear. Recent research has illuminated the pathophysiology of RA, underscoring the central role of T cell interactions with antigen-presenting cells (Sharma et al., 2012). To further our understanding and potential treatment options, studies have delved into the use of MNPs conjugated with polyclonal anti-interleukin antibodies (Sherr, 2004). The results are a testament to the success of this conjugation, with microscopic examinations and spectrophotometric analyses affirming specific antibody binding to nanoparticles. The noteworthy shift in absorption peak from 410 to 430 nm in the spectrophotometric analysis is indicative of effective antibody conjugation, holding immense promise for applications in targeted drug delivery and diagnostics (Pramoolsinsap et al., 1994).

Amidst these developments, IL-2 has emerged as a key player, with implications for conditions like type 2 diabetes and RA. Studies have shown that IL-2, particularly low-dose IL-2 (ld-IL2), possesses therapeutic potential due to its ability to expand and activate regulatory T cells (Tregs) (Prasad, 1985). Notably, RA patients have displayed a connection between blood IL-2 levels and insulin sensitivity, suggesting a role for IL-2 in the development of insulin resistance, a primary metabolic concern in RA. This association may be attributed to IL-2's impact on immune function, primarily the generation of cytolytic T cells (Prasad, 1985).

Moreover, studies have shown that the rise in IL-2 production can lead to an imbalance between T helper 17 (Th17) and Treg cells, correlated with the rheumatoid titer factor and increased disease severity. This outcome underscores the role of IL-2 in immunological dysregulation, contributing to a decrease in Treg cells and an increase in effector T cells and natural killer cells. In response, low-dose interleukin-2 injections have shown promise in treating RA by promoting Treg cell proliferation (Prasad, 2001a).

In balancing the potential therapeutic benefits with concerns regarding immune system activation, preliminary research endeavors aim to block IL-2 production, a crucial element in RA pathogenesis. Notably, mouse-generated antibodies directed against IL-2 have been explored using enzyme-linked immunosorbent assay (ELISA) and western blotting, revealing a promising relationship. This points to the potential utilization of IL-2/mAb complexes in animal and human therapeutic trials, as well as the development of novel medicines. These research efforts offer a glimpse into the future of safer and more effective immunoregulation strategies, notably employing IL-2 as a critical tool (Prasad, 2001b).

The study found significant differences in Anti-IL-2 IgG and highly significant differences in Anti-IL-2 IgA levels between RA patients and controls. These antibodies were more prevalent in RA patients. ACPA levels did not significantly differ. When comparing RA patients with and without erosive disease, Anti-Interleukin antibodies were significant markers, while ACPA was not. RA patients under biological treatment showed significant differences in Anti-Interleukin antibodies, indicating their potential as treatment response markers. This data suggests that Anti-Interleukin antibodies may have diagnostic and prognostic value in RA.

Simultaneously, the transition detailed in the provided table, moving from oral methotrexate (MTX) to subcutaneous local manufactured MTX, aligns with existing literature and underscores the advantages of subcutaneous MTX administration. Multiple studies and clinical trials have consistently demonstrated the benefits of subcutaneous MTX, including enhanced bioavailability, faster response, and potentially reduced gastrointestinal side effects compared to oral MTX (Prasad, 2001b).

A crucial parameter supporting this transition is bioavailability, with studies revealing that oral MTX's bioavailability is constrained, especially at doses exceeding 15mg per week. In contrast, subcutaneous MTX maintains a linear, dose-dependent increase in bioavailability (Quer et al., 2017). This feature is pivotal in understanding why subcutaneous MTX can be more effective, providing higher drug exposure even at doses exceeding 24mg per week. The consistent elevation of the area under the plasma drug concentration-time curve (AUC0–t) with subcutaneous MTX further substantiates the argument for bioavailability.

Additionally, subcutaneous MTX injections have been linked to increased levels of long-chain MTX polyglutamates, associated with reduced disease activity and improved MTX response in patients. This transition fosters rapid increases in long-chain MTX Glu levels, enhancing cellular retention and drug availability and explaining the expedited response to subcutaneous MTX (Ramadori et al., 1985; Rani et al., 2018).

Moreover, in addressing gastrointestinal side effects, the shift from oral to subcutaneous MTX appears to alleviate these concerns. Studies have consistently reported a lower incidence of adverse gastrointestinal effects, particularly nausea and diarrhea, with subcutaneous MTX. This highlights the potential advantage of subcutaneous administration in mitigating these specific side effects, often associated with oral MTX (Rani et al., 2018).

In summary, the provided table aligns with existing literature, emphasizing the benefits of subcutaneous MTX administration over oral MTX. This transition enhances therapeutic precision by offering enhanced bioavailability, quicker responses, and potentially fewer gastrointestinal side effects. These considerations reflect the current scientific understanding and clinical practice in the field of MTX therapy.

Conclusion

In light of the challenges many RA patients face with existing medications, the development of a novel therapeutic approach is imperative. Interleukin-2 (IL-2) holds significant promise in immune system regulation, effectively governing both immune stimulation and suppression. Notably, anti-IL-2 antibodies have demonstrated a favorable association with IL-2. Moreover, elevated IL-2 expression has been linked to a reduction in regulatory T cells (Tregs), a phenomenon contributing to the development of RA. Consequently, the effective reduction of IL-2 expression in individuals with RA presents a promising avenue for treatment.

Simultaneously, subcutaneous MTX offers notable advantages over its oral counterpart, particularly in terms of decreasing gastrointestinal infections and alleviating illness in RA patients. The incorporation of subcutaneous MTX as a preferred treatment method in healthcare settings stands to significantly improve the quality of life for RA patients, addressing both the symptoms and the underlying disease.

In conclusion, the synergy of IL-2-focused therapies and the transition to subcutaneous MTX represents a compelling path forward in the management of RA. These combined approaches offer a more effective means of controlling illness, reducing pain, and enhancing the overall well-being of RA patients. It is essential that these advancements are adopted in clinical practice to provide much-needed relief for those suffering from RA.

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Data Availability: All the generated and analyzed data is present in this manuscript.

Competent of Interest: There is no competing interest between authors.

Ethical Statement

This study was conducted according to guideline of ARRIVE and UMT and superior university –Azra Naheed Hospital) Ethical Committee (Approval Number 178/22) while rat was used in the experiment for the antibodies work after obtaining ethical approval from University of Management and Technology Ethical Board while no rat was harmed and killed or slaughter during this work. No animal nor human was harmed during this study. While consent was obtained from all the patients in written at Shalimar Hospital during collecting data.

Informed Consent

All the experimental protocols were approved by the institute (and superior university -Azra Naheed hospital) and the informed consent was obtained from all the RA patients before giving them dose of MTX in injection (they were already taking this dose previously orally). Patient's disease history was also discussed and monitored. The patient's consent was taken by using all the inclusion and exclusion criteria set by Helsinki institute. All the experimental protocols were approved, and all the consents were approved and evaluated by the institute (UMT and superior university-Azra Naheed hospital) where study was conducted and subjects were taken for injecting MTX.

Patients (humans) for the Study

All the experimental protocols were approved by the UMT and superior university -Azra Naheed hospital. The clear informed consent was taken before injection of MTX keeping all the inclusion and exclusion criteria set by finding demographics and disease history according to Helsinki institute. The patients were receiving MTX orally for many years and are now ready for injection. No human (subject) with rheumatoid arthritis injection with MTX was harmed or got any type of allergy in this study, or during clinical trial.

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