

6 Discussion

The prevalence of cardiovascular complications (CVD) in patients suffering from Rheumatoid arthritis (RA) is one of the major concerns today which is associated with both morbidity and mortality^(127, 128). There are two main reasons of occurrence of CVD in RA: The first being inflammation driven by receptor activation responsible to initiate multiple pathways at a same time in different biological systems and works as underpinning factor in both the diseases and second one is the side effects and drawbacks of existing therapies^(102, 129).

In preclinical scenario also, RA is protracted disease as there is no such animal model developed till date which gives a complete insight of CVD in RA. The current study was aimed to target these two problem statements. At primary stage animal model replicating CVD in RA was developed using Lipopolysachcharide (LPS) and High Fat Diet (HFD) with CFA and collagen, here aim of model creation was to activate highly expressed proteins/receptors (TLR-4) in the synovial lining and sub lining layer of RA synovium and cardiac muscles via endotoxemia, cell infiltration and stimulation of cytokines (TNF- α , IL-6, IL-8, and NF- κ B) responsible for CVD in RA^(15, 94). As this model was new of its kind, it was validated on certain parameters to check its reproducibility and resemblance with human disease. At second stage three herbs; *Nigella sativa* seed, *Carica papaya* seed and *Momordica charantia* seed were chosen for formulation development in present study due to their common pharmacological properties which synergistically acts as anti arthritic, Immounomodulatory and cardioprotective drugs^(78, 130, 131). These herbs were evaluated as preventive therapy in the developed models on the basis of principles of Ayurveda, where *Amavata* (Rheumatoid Arthritis) is considered as metabolic disease. As existing therapies for RA have risk associated with cardiovascular complications, this new synergistic combination also relieve cardio vascular complication along with existing Rheumatoid Arthritis.

As this study was conducted with two different goals of development and validation for novel condition of CVD in RA using rats and the treatment of this situation with herbal components the discussion is also done on this basis.

Here, *Bovine collagen type II* was selected over **Adjuvant induced model (CFA)** because when rats sensitized with CFA, rapid explosive onset of highly erosive polyarthritis occurs and activation of Rheumatoid Factor (RF) is also dependent on *HLA-DRB* gene activation⁽¹³²⁾.

Additionally CFA followed inflammatory mediated pathway initiate by *HSP-65* (heat shock protein 65) which is unable to mimic bony ankylosis and extra-articular manifestations⁽⁵⁹⁾. On the other hand bovine collagen type-II (CIA) induced rat models suggests symmetrical joint involvement, peripheral joints involvement, persistent joint inflammation, synovial hyperplasia, inflammatory cell infiltration, marginal erosions, presence of rheumatoid factor and anti-collagen antibodies, genetically regulated by MHC and non-MHC genes and these models are responsive to most therapies effective in RA⁽¹³²⁾. In present study also the rats sensitized with collagen were noticed early disease onset with severe inflammation and secondary lesions which was persistent with atherogenic stimulation also.

In the current study the outcomes of physical, biochemical and perceptible indicators suggesting the higher impact of collagen in terms of disease severity and disease onset.

Lipopolysaccharide (LPS) was incorporated as secondary inducers which is an endotoxine and activator of TLR-4 receptors with cytokine overproduction at the inflammatory site^(103, 133). The dose of LPS was tapered from lower to higher in different groups in micrograms just to add the extra immune an inflammatory insult and to prevent the sepsis like and hepatic damage.

Here, incorporation of LPS suggested giving the initiation to multiple pathways associated with RA to generate CVD in RA on the basis of result obtained.

Diet modification (High Fat Diet)⁽⁹¹⁾ was selected for two reasons; primarily for addition of fat content in animals to give a high lipid content environment to develop cardiovascular complications and these fatty contents when increased in body they alter the metabolic functions and initiates the auto immune events in the form of metabolic dysbiosis and leaky gut⁽¹⁰³⁾. This leaky gut is associated with the alteration of gut flora which secretes the LPS in such environment. When a little boost of LPS is also provided by outside, the over secretion of LPS creates the inflammatory, immune and other pathway activations which are cross linked to extra organ damages majorly cardiovascular complications⁽¹³⁴⁾.

The models were developed for RA and cardiovascular complications in RA with different inducers and prevalidation studies were done on the basis of disease indicators. In this study paw volume, arthritic score, arthritic index, neutrophile count, CRP, ESR, RF, TNF- α , NFkB, Anti- CCP, X-ray were specifically evaluated for RA markers.

Homocystein, TLR-4 were selected as confirmatory tests for progression of extra organ manifestations in terms of CVD in RA.

Cardiovascular complications were estimated after high fat incorporation in animals with RA induction and this addition causes metabolic dysbiosis which was evaluated with body weight, TG, TC, LDL, HDL estimations with RA indicators. Here cardiac risk ratio and atherogenic indicators are major criteria for assessment of cardiovascular complications in RA with confirmatory tests as histopathology of vastus medialis and biceps femoris muscle along with bone histology for confirmation of RA.

The individual models for RA and CVD in RA were compared for mentioned indicators of disease progression and severity where,

*The models which proved to give human resemblance were validated via Framework to Identify Models for Disease (FIMD)⁽¹²⁴⁾ to insure the internal and external validation. **Model I (CFA 0.1ml+LPS 10µg/ml)** secured **moderately validated score 64%** with **highest uncertainty factor 36%** among final four groups for RA generation; **model I (CFA 0.1ml)** **model II (CFA 0.1ml+LPS 10µg/ml)**, **model III (CIA 0.1ml)** and **model IV (CIA 0.1ml+LPS 10µg/ml)**.*

Model II (CIA 0.1ml+LPS 10µg/ml) was compared firstly to get the significant model for RA only with model I. Where **model II (CIA 0.1ml+LPS 10µg/ml)** lied in **highly validated model (82% validity score)** which shows the maximum clinical resemblance for RA with clinical situations. Moreover the **uncertainty factor** for this model was **18%** which gives the accounts of the domain which are not common. The **similarity factors** was also calculated between these two models which shows the points in domains where both the models having **40% similar representations of RA.**

In second stage the **model I (CFA 0.1ml+LPS 10µg/ml)**, **model II (CIA 0.1ml+LPS 10µg/ml)** **model III (CFA 0.1 ml+LPS 10µg/ml +HFD)** and **model IV (CIA 0.1 ml+LPS 10µg/ml +HFD)** were compared for optimizing the interconnecting parameters which are responsible for progression of cardiovascular complications in existing RA.

In final comparison **model IV (CIA 0.1 ml+LPS 10µg/ml +HFD)** lied in **highly validated model (95% validity score)** and **uncertainty factor** was **5%** which proves the **model IV** has **maximum resemblance with clinical situations.** All these four models were having some similar domains as similarity factor among all the groups is **14%.**

On the basis of above interpretations we can conclude that the **model IV (CIA 0.1 ml+LPS 10µg/ml +HFD)** is validated and optimized model for cardiovascular complications in stable RA which gives the maximum insight of clinical situation on the basis of FIMD validation method.

After model development and validation, extracts of selected herbs were evaluated in final validated models on certain parameters to find the effectiveness of the inducing agents in terms of disease generation and in terms of prevention of disease by using the extracts of selected herbs in this evaluation following estimations were done-

The first sign of biological insult is **Inflammation and edema** which occurs due to protective mechanism of body defense against the foreign entities (antigen, bacteria and virus)^(25, 43). RA is one such disease which occurs due to erosion of bone, depletion of fluid and finally edema and inflammation in and around the affected joints which is clinically the first sign of occurrence of RA to leads joint pain, stiffness and walking disabilities⁽⁴³⁾.

Paw volume in study was performed on day 1, 3, 5, 7, 11, 14, 17, 21, 28 for CFA groups and extended for day 35 and 42 for collagen induced models. Among CFA induced groups, model I showed 275% increase in paw volume in respect to non diseased animals.

The paw volume of final selected group from collagen induced RA; Model II showed the 296% increase on day 28 which was higher than CFA induction. The group II and IV were further compared for paw volume on day 42 where, group IV showed 775% increase in disease severity.

*The comparison of both the inducing agents showed that the collagen induction had early onset of disease with severity. On the other hand the treatment control group (NSAE 200mg/kg+CPAE50mg/kg+MCAE400mg/kg) showed the 100% decrease and recovery in paw volume at the end of study which shows the anti inflammatory and anti arthritic effects of combination of aqueous extracts of *Nigella sativa*, *Carica papaya* and *Momordica charantia* seed.*

Arthritic scoring (one of the measures to check the severity) in terms of primary lesions (day 5) appears due to high grade inflammation in body and secondary lesions (day 21) appears due to immunological drive in response to disease progression. Inflammation, wounds, increased temperature, secretions and nodules on body can be counted in these lesions. These abnormal signs denote the disability with increase in severity of disease⁽¹³⁵⁾.

Normal control group was not sensitized with any adjuvant so the score of this group was 0 and groups were compared in between the models on the basis of days.

Scoring done by blind observer showed that there was 48% increase in scores of day 5 and 21 in model control I in terms of lesions appeared. In model control group IV the increase

was 63% increase in secondary lesions. The standard control group showed decrease of score by -18% and the treatment control showed decrease of -75%

The comparison of arthritic score suggests the anti inflammatory and anti arthritic activity of combination.

Arthritic index is one of the measures which show the cumulative effects of disease progression in terms of high grade inflammation, bone deformities and disabilities generated due to amalgam of inflammatory and immune responses.

This is one of the basic estimations on lab scale to check the disease severity in animals with physical measures along with biochemical confirmations.

Again Arthritic index was compared with treatment control groups as normal animals were not sensitized with inducing agents and their index was 0 throughout the study⁽¹³⁵⁾.

There was 682% increase in model control IV as compared to treatment control group. The standard control group also showed 508% increase in arthritic index. And CFA induced groups also showed the 518% increase in arthritic index.

The results of arthritic index indicating that there was a severe disease onset in collagen induced groups as compared to CFA groups and the treatment combination has potential to reduce high grade inflammation.

C-RP is a systemic and specific inflammatory marker generated by liver in micro inflammatory environments. In RA, as inflammatory state persists for longer period which chronic events these markers can lead the immune responses to link atherogenic conditions⁽¹³⁶⁾. In this study CRP was measured on day 1, 7, 14, 21, 28 in CFA sensitized groups and in collagen induced groups it was further extended to 35, 42.

In model control I 234% and model control II showed 250% increase in CRP as compared to normal control group the final selected model IV for CVD in RA 284% increase in CRP was observed. The standard control group also showed the 186% increase in CRP levels.

The combination T2 showed the 98% decrease in CRP levels in preventive treatment which shows the effect of combination on inflammatory markers.

The CRP data indicates that the treatment control group is effective to give anti inflammatory effects on RA and CVD.

ESR is one of the acute phase proteins which activates during inflammatory conditions in body with CRP. In clinical researches it has been revealed that the patients having RA and increased ESR levels up to six months are more prone to heart diseases in near future⁽¹³⁷⁾.

This inflammatory marker is one of the key initiators to link inflammation with immune responses.

In the current study ESR was measured on weekly basis and the initial results of day 1 when compared with day 7 were nonsignificant, but as inflammation and disease progression increased model control groups showed significant increase in ESR. The model control IV showed the higher sedimentation rates by 1415 % increase of as compared to disease severity increase in standard groups 186%. Treatment combination T2 showed -69% decreases in ESR.

In RA environmental factors affects genetically susceptible persons and activate the immune responses by alteration of self peptides citrullination responsible for generation of self destructing autoantibody (Anti-CCP/ ACCP/ ACPA) which expressed in 75% of RA patients and this is the major factor which can distinguish RA with osteoarthritis.

Anti- CCP was estimated in this study for two reasons; to confirm that the developed model represents RA and to insure that immunological intervention is involved in the model^(14,61).

The results suggest that the ACPA activation was generated in all models. The activation of ACPA in CFA control showed 221% increase in group I and group II showed 317% increase. On the other hand collagen induced groups showed 909% increase in Anti-CCP which indicates high immune intervention. Standard control group also showed decrease 40% decrease in ACPA. Treatment group T2 showed the highest impact on ACPA with 100% decrease in ACPA which showed that the combination is effective on immune responses on CVD in RA.

Rheumatoid Factor (RF) is one of the markers in RA which has dual connection with environmental as well as genetic factors. In clinical situations it was seen very commonly that some patients who are having stable RA can show the seronegative results for RF as well as some time the patients who does not show any evidences of deformities can show the seropositive responses of RA. Genetic factors can play dual role for prevention or for activation of the certain characteristic in body. The major player in this category is Human Leukocyte Antigen (HLA) which is auto reactive lymphocytes and they are one of the most important parts of central as well as peripheral immune system⁽⁶¹⁾.

In model control group RF was increased in collagen induced group, highest 1103% increase seen in model group IV which confirms the induction of RA in animals. CFA control groups

the rise in RF was seen by 51% increase and 97% increase these expressions may be related with genetic susceptibility of HLA-DRB gene.

The expressions of RF in treatment control T2 decreased by 84% which shows the effectiveness of the selected combination as Anti arthritic drug.

Homocysteine (Hyc) is one of the factors which denote the extra organ manifestations, this biomarker generate in body via Methylation of methionine in presence of sulfur and vita B12 in liver. Hyc is associated with so many different biomarkers which leads the free radical formation, endothelial dysfunction and Thromboxane synthesis^(97, 138).

The current investigation was attempted to incorporate cardiovascular complications in the form of atherosclerosis in rats where Hyc is one of the markers to estimate the extra organ manifestations.

Model I (CFA+ LPS) showed elevations in Hyc levels by 52% increase the model control groups for RA and CVD group IV showed the Hyc levels by 233% increase. Whereas treatment groups showed the low level expression of homocysteine among which treatment T2 was found to decrease Hyc levels by 96% decrease.

These results showed that the combination of selected herbs is effective in prevention of extra organ manifestations of CVD in RA.

Proinflammatory cytokines such as **interleukin (IL-1, IL-6), Tumor Necrosis Factor-alpha (TNF- α) and NF- κ B** are central mediators in parallel damage in synovium and arteries due to endothelial damage and highly expressed T cell and B cells. In result endotoxemia, gut permeability microbial burden and cell infiltration are stimulated which results in impaired Immunity^(15, 66).

Interlukine-6 (IL-6) is the major cytokine release in synovial membrane in inflammatory conditions mediated via cell infiltration in chronic inflammatory conditions^(10, 139). It is also one of the common factors which work similarly in animal and humans for articular and systemic manifestations for RA as well as atherogenic conditions.

In this study there was increase in IL-6 in model control group by 275% as compared to normal control and Test group T2 showed 88% decrease in IL-6 as compared to model control group (IV). Standard control group also showed 93% decrease in IL-6 levels which suggest the effectiveness of Mtx on IL-6 better than the treatment control group.

TNF- α is another proinflammatory cytokine responsible for the production and secretion of autoantibodies in RA with other receptors involved in the pathogenesis. TNF- α is responsible for production of monocytes, macrophages, B cells T cells and fibroblasts. TNF- α is also responsible for the production of IL-6, IL-1 and GM-CSF in synovial membrane which are stimuli of extra organ damage in vascular endothelial damage in stable RA⁽¹⁴⁰⁾.

There was a 468 % increase in TNF- α was seen in model control group IV as compared to normal control. Test group T2 showed decrease of 79% in TNF- α compared to model control groups. Standard group showed 50% decrease in TNF- α level as compared to model control. These results suggest that the selected combination is effective on immunological parameters similar to standard drug methotraxate.

NF- κ B is equally important biomarker in RA as well atherosclerosis. TNF-receptors (TNF-R) activate a family of adapter proteins called TNF-R associated factors (TRAF) which activate downstream signaling pathways including nuclear factor κ B (NF- κ B)-mediated transduction mechanisms. NF- κ B is normally found in the cytoplasm in an inactive complex with I κ B⁽¹⁴¹⁾. Many of the products of NF- κ B inducible genes are involved in inflammation and immune responses. These genes encode adhesion molecules, cytokines, growth factors, cytokine receptors, immunoregulatory molecules, and acute phase proteins.

In the present study the activation of NF- κ B was observed in model control group by 693% increase as compared to normal control. Test group also showed the decrease of 160%. Standard control showed a moderate elevation on this biomarker by 81% which suggests the better effectiveness of Mtx. on immuno components.

Lipid profile or elevations of lipid components responsible for metabolic disorder as well as obesity are one of the major components to be regulated in RA patients. As in RA there is a persistently high level of inflammation which is an associated risk of developing cardiovascular diseases. If inflammation is high grade and chronic it leads to alteration of HDL structure due to oxidative stress and also causes reduction of apolipoproteins. In clinical trials also it has been observed that RA patients have low levels of paraoxonase-1 which is an antioxidant enzyme associated with HDL. All these factors causes the interference is natural protective effect of HDL as anti-inflammatory, antioxidant and cardioprotective⁽¹⁴²⁾.

In clinical trials it has been also observed that Total cholesterol, Triglycerides, VLDL & LDL were increased but serum HDL decreased among RA groups.⁽¹⁴³⁾

Triglyceride levels in model control group were found to generate 326% increase in TG levels, HFD fed groups showed 366% increase in TG levels. The standard control group showed only 30 % decrease in TG levels which shows that Mtx has very low effects on lipid profile. Whereas the treatment combination showed 90% decrease in TG levels which suggests the positive effects on triglyceride levels.

Total cholesterol was also increased gradually in RA with CVD models, the model showed 167% increase in cholesterol levels in CVD in RA model. The standard control group also showed 182% increase in cholesterol levels. The treatment groups showed the 99% decrease in cholesterol levels in CVD with RA.

HDL levels were decreased in model control animals by 80% decrease which suggests the increase in atherogenic events in model control groups. The treatment control groups showed 70% increase in HDL levels which suggest the protective effects of combination for CVD in RA.

Cardiac Risk Ratio in model control group showed 438% increase which is an indicator for atherogenicity in developed model. The treatment control group showed 80% decrease in this mediator which shows the protective effects of treatment combination.

Atherogenic index were also found increased in model control group by 358% increase and it was found to be decreased in treatment control groups by 57% which suggests the effectiveness of selected combination in CVD in RA. Standard control group also showed elevation in this marker by 284% which suggests the initiation of cardiovascular complications in stable RA.

Radiographic and histopathological examinations are confirmatory tests for assessment of disease progression as well as effectiveness of treatment control groups.

The edema and bone deformities were clearly seen in model control animals which suggests the stable RA conditions in rats was developed successfully. Moreover the symmetric progression of diseases confirms the RA. The whole body X-Ray of animals also suggests the deformities and bone destruction in animals of model control group.

Histological changes also confirm the Pannus formation, cell infiltration and destruction of synovial membrane in paw and on the other hand histopathology of heart, shortening of the muscle fibre of vastus medialis and biceps Femoris muscles and sections of aorta suggests the confirmation of atherogenic activities in developed models. On the other hand the selected herbs proved to have potential to prevent this damage in terms of RA as well as

cardiovascular initiation in RA which suggests that the hypothesized study was proved the positive results with right path of disease generation and the treatment part as “Pharmacological evaluation of some herbs in experimentally induced Rheumatoid Arthritis along with cardiovascular complications” when performed with combination of select nutraceuticals; Nigella sativa seed, Carica papaya seed and Momordica charantia seed aqueous extract in combination (NSAE 200mg/kg, CPAE 50mg/kg MCAE 400mg/kg) showed the ameliorative effects on physical, perceptible as well as inflammatory and immune responses on RA as well as the progression of factors responsible for cardiovascular complications.