ORIGINAL ARTICLE

Methotrexate and theaflavin-3, 3′-digallate synergistically restore the balance between apoptosis and autophagy in synovial fibroblast of RA: an ex vivo approach with cultured human RA FLS

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Abstract
Background  Imbalance between apoptosis and autophagy in fibroblast-like synoviocytes (FLS) is one of the pathogenic mechanisms responsible for their abnormal proliferation in rheumatoid arthritis (RA). Methotrexate (MTX) demonstrated limited efficacy in amending this imbalance in fluid-derived (fd)-FLS. The active compound of black tea Theaflavin 3,3′-digallate (TF3) may be effective in restoring apoptosis–autophagy imbalance in (fd)-FLS. The combined effect of MTX + TF3 upon the same is yet to be elucidated.

Objective  To evaluate the effect of MTX + TF3 on fd-FLS to induce apoptosis and inhibit autophagy through Endoplasmic Reticulum (ER) stress-mediated pathways.

Methods  FLS from synovial fluid of 11 RA and 10 osteoarthritis patients were cultured after treatment with MTX/TF3 or a combination of MTX (125 nM) and TF3 (10 µM) and the following parameters were evaluated. C-reactive protein, cytokines (TNF-α, IL-6), angiogenic markers were quantified by ELISA. fd-FLS viability was determined by MTT assay and apoptosis by flow cytometry. ER stress markers were estimated by RT-PCR (IRE1A, spliced-XBP-1) and immunoblotting (Grp78, Hsp70, CHOP, HIF-1α). Immunoblot studies were done to evaluate apoptotic (Bcl-2, Bax, Caspases) and autophagic (Beclin1, LC3b, p62) proteins.

Results  MTX (IC25) and TF3 (IC50) both in single doses could down-regulate the levels of pro-inflammatory and angiogenic markers. Combinatorial treatment modulated autophagosomal proteins in fd-FLS and induced apoptosis by regulating ER stress response.

Conclusion  Disruption in homeostasis between apoptosis and autophagy in fd-FLS might be an underlying phenomenon in the progression of pathophysiology in RA. Co-administration of MTX + TF3 successfully restored the homeostasis by inducing apoptosis.

Keywords  Rheumatoid arthritis · Fibroblast-like synoviocytes · Hypoxia · Angiogenesis · Endoplasmic reticulum stress · Apoptosis/autophagy imbalance · TF3 · MTX

Abbreviations
ANG-1  Angiopoietin 1
ACR/EULAR  American College of Rheumatology/European League Against Rheumatism
ANOVA  Analysis of variance
Caspase  Cysteine-aspartic proteases
CRP  C-reactive protein
CHOP  C/EBP homologous protein
cDNA  Complementary DNA
DMARDs  Disease-modifying anti-rheumatic drugs
DEPC  Diethyl pyrocarbonate
ELISA  Enzyme-linked immunosorbent assay
Introduction

RA, a systemic, chronic autoimmune disorder, mainly associated with inflammation and pain in peripheral joints. In RA, there is hyperplasia of the synovial cell lining as well as pannus formation, termed as ‘synovitis’ (Guo et al. 2018). Infiltration of synovial fluid-derived fibroblast cells (fd-FLS) disrupts the synovium lining that leads to the perpetuation of disease pathogenesis and progression (Stebulis et al. 2005). Within the synovium, increased pro-inflammatory cytokine milieu (TNF-α, IL-6) induces cellular proliferation resulting in depletion of oxygen and facilitates a hypoxic microenvironment. This hypoxic stress eventually triggers the expressions of HIF-1α, VEGF, and angiogenic factors like ANG-1 (Elshabrawy et al. 2015; Quinonez-Flores et al. 2016).

In the endoplasmic reticulum (ER), integral homeostasis is maintained by intricately coordinated adaptive interactions between unfolded protein responses (UPRs) and ER-associated degradations (ERAD) of accumulated misfolded proteins (Park et al. 2014). An imbalance in homeostasis within ER increases the rate of UPR, subsequently ER stress (Guzel et al. 2017). GRP78, a well-recognized molecular chaperon, is known to be activated during ER stress and consequently binds to misfolded proteins (Yoo et al. 2012). It has been previously reported that hypoxic stress-induced proteins can stimulate ER stress responses and their respective downstream signaling proteins in fd-FLS (Park et al. 2014). During hypoxic and angiogenic stress, a substantial amount of various proteins get misfolded/unfolded and accumulated, which possibly trigger apoptosis of fd-FLS in patients with RA (Park et al. 2014). On the contrary, despite being modulated by increased ER stress, delayed apoptosis of fd-FLS is also reported (Kabala et al. 2017). Autophagy is a highly conserved homeostatic mechanism that regulates the degradation of damaged cytosolic constituents (Chun and Kim 2018), and it also induces apoptosis, implicating a dual role of autophagosomal machinery (Kato et al. 2014). In comparison to patients with OA, higher ER stress-mediated autophagy activation in RA demonstrated more apoptotic resistance in fd-FLS (Xu et al. 2015).

MTX is a conventional DMARD used in the treatment of patients with RA. The efficacy of MTX varies among patients, and approximately 50% of the recipients showed negligible responses (Yu et al. 2018). Despite being toxic, a low dose of MTX is proven to be beneficial in inducing apoptosis in synovial cells (Herman et al. 2005). The effect of MTX upon the proliferation of monocytes and its inadequacy in halting fd-FLS proliferation has been previously reported (Bergstrom et al. 2018). TF3 is an active compound of black tea. TF3 has the potential to prevent inflammation and induce apoptosis in numerous cell types both in malignancy and in a mouse model of RA (Liu and Li 2019), as well as in inflammatory diseases and cancer (Tu et al. 2016).

Based on this background, our study was aimed to evaluate the effect of TF3 (anti-inflammatory and pro-apoptotic), low-dose MTX alone, and in combination (TF3 + MTX) upon cell proliferation/viability, markers of hypoxia, angiogenesis, the status of apoptosis, and associated ER stress-mediated autophagy in fd-FLS in patients with RA.

Materials and methods

Study design

The study population included patients with OA and RA with effusion in at least one knee joint. All RA patients
fulfilled the ACR/EULAR classification criteria for RA 2010 (Aletaha et al. 2010). Patients were recruited from the outpatient department of Clinical Immunology and Rheumatology of The Institute Of Post-Graduate Medical Education And Research (IPGME&R), Kolkata, who have already been administered with DMARDs with or without NSAIDs. Patients with any other rheumatological disease, diabetes mellitus, hypertension, hypothyroidism, body mass index ≥ 25, pregnancy, renal and/or hepatic impairment, receiving steroids, or any antioxidants were excluded. This study was approved by the Institutional Ethics Committee (No: Inst/IEC/24.02.2014). Informed consent was obtained from all participants. Baseline demographic and clinical data were collected from all the patients. Approximately 10 ml of synovial fluid was aspirated from the knee joint of the participants maintaining strict aseptic measures.

Cell culture and reagents

Isolated cells were cultured in DMEM (Dulbecco’s Modified Eagle Medium, Cat no-D5648 Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum/FBS (Invitrogen, Rockford, IL, USA), 1% penicillin–streptomycin (10,000 U/ml, Cat no-15140122, Invitrogen) at 37 °C with 5% CO2 in a humidified incubator. Theaflavin-3, 3′-digallate/TF3 [Cat no-922223], and Methotrexate/MTX [Cat no-M8407] were purchased from BD, Biosciences. Anti-LC3b antibody (ab51520) was procured from Abcam. The VEGF-A and ANG-1 ELISA Kits [(#ELH-VEGF-1) and (#IQH-Angiopoietin1-1)] were procured from (Ray Biotech, Norcross, GA). For detection of apoptosis FITC Annexin V Apoptosis Detection Kit, I [Cat no-556547] was used from BD, Biosciences.

Culture of synovial fd-FLS

SF from patients with RA and OA was centrifuged at 3000 rpm for 10 min. The cell pellet was re-suspended in DMEM supplemented with 10% FBS, 2 mM l-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and plated in 25cm2 flasks. After 24 h, non-adherent cells were discarded. Spindle-shaped cells were observed after the third passage.

Characterization of fd-FLS by immunophenotyping

Cultured fd-FLS cells (5 × 10^5 cells/ml) were incubated with FITC conjugated CD90 [BD Biosciences Cat no: 555595] according to the manufacturer’s protocol. Cells were incubated for 20 min in dark at room temperature (20–25 °C) followed by a one-time wash with PBS (1 ×) at 4000 rpm for 5 min. The cell pellet was then resuspended with 400 µl of PBS and subjected for FACS analysis (FACS Verse, BD Bioscience San Jose, CA, USA) and analyzed in FACSuite software.

Extracellular cytokines measurement by ELISA

Extra-cellular levels of pro-inflammatory cytokines (TNF-α, IL-6), angiogenic markers (VEGF-A and ANG-1), and inflammatory marker (CRP) were measured from cell-free supernatant of cultured fd-FLS following manufacturer’s protocol.

Cell viability assay

The pro-apoptotic activity of TF3 alone and in combination with MTX was evaluated in fd-FLS obtained from RA patients by MTT assay (Dutta et al. 2005). Briefly, 5 × 10^4 cells/ml were seeded in 96-well plates in DMEM medium (supplemented with 10% FBS and antibiotics) and incubated with TF3 (0–50 µM), respectively for 48 h. For combinatorial treatment, one dose of MTX (125 nM) was incubated with variable dose of TF3 (0–30 µM) for 24 h. After treatment incubation, fd-FLS viability was measured using 3-(4, 5 dimethylthiazole-2-yl)-2, 5-diphenyloxetrazolium bromide, and the optical density (OD) was measured at 492 nm in a microplate reader (Byonoy absorbance 96 plate reader). The results were expressed at IC50 values, i.e. the concentration that inhibited 50% of cell growth, enumerated by graphic extrapolation using GraphPad PRISM software (version 5).

Analysis of apoptosis in cultured fd-FLS by flow cytometry

Isolated cells were treated for 24 h with both MTX and TF3, followed by washing twice with 1xPBS. Cells (1 × 10^6 cells/ml) were dissolved in 1 x PBS. Cells were then centrifuged for (10 min, 3000 rpm) and the cell pellet was resuspended in 100 µl of (1 ×) annexin V-binding buffer and kept for 10 min in dark at room temperature. FITC conjugated annexin V and propidium iodide (PI) were added, kept for 15 min and 5 min, respectively, in dark at room temperature. Cells were then finally resuspended in 400 µl of annexin V binding buffer. Cells were acquired (events: 5000) in FACS
stored at −20 °C for further processing.

The mixture was taken out and kept on ice for 5–10 min and then incubated again at 70 °C for 10 min. After that, the mixture was allowed to stand for 5 min at room temperature. Next, 200 μl of chloroform was added and shaken vigorously for 10 min. Then, the mixture was allowed to stand at room temperature (20–25 °C) for 2–5 min and was again centrifuged at 10,000 rpm for 10 min. The exact upper aqueous phase was then transferred to a fresh tube followed by the addition of 0.5 ml of isopropanol and was again subjected to vigorous shaking for 10 min. The mixture was then kept at room temperature for 10 min. Centrifugation was carried out at 10,000 rpm for 10 min at 4 °C to settle down the pellet from the mixture. The RNA pellet was collected, washed with 75% ethanol followed by centrifugation at 10,000 rpm, and allowed to air dry at room temperature. Finally, the dried RNA pellet was re-suspended in DEPC water (20 μl) and quantified in a nano-drop spectrophotometer (Thermo Scientific, USA). Approximately, 1 μg of cDNA was prepared from isolated RNA with a total volume of 11.5 μl (RNA + DEPC water). Next, oligo dT (1 μl) was added and incubated at 70 °C for 5 min inside the PCR machine (Applied Biosystems, USA). The mixture was taken out and placed in chilled ice for 5 min followed by the addition of PCR master mix. The mixture was given a short vortex to mix well followed by incubation at 37 °C for 5 min. Then, 1 μl of reverse transcriptase was added and incubated at 42 °C for 60 min inside the PCR machine. Finally, the mixture was again incubated at 70 °C for 10 min after that the mixture was taken out and kept on ice for 5–10 min and stored at −20 °C for further processing.

**RNA isolation and cDNA synthesis**

Total RNA was extracted from SF-derived synovial fibroblast cells using 300 μl TRIzol™ (Invitrogen, Carlsbad, CA, USA) at a ratio of (1:1). Cells were slightly flush and stored overnight at −20 °C. On the next day, the mixture was centrifuged (10,000 rpm for 5 min at 4 °C). The clear supernatants were transferred to a fresh microcentrifuge tube and allowed to stand for 5 min at room temperature. Next, 200 μl of chloroform was added and shaken vigorously for 10 min. Then, the mixture was allowed to stand at room temperature (20–25 °C) for 2–5 min and was again centrifuged at 10,000 rpm for 10 min. The exact upper aqueous phase was then transferred to a fresh tube followed by the addition of 0.5 ml of isopropanol and was again subjected to vigorous shaking for 10 min. The mixture was then kept at room temperature for 10 min. Centrifugation was carried out at 10,000 rpm for 10 min at 4 °C to settle down the pellet from the mixture. The RNA pellet was collected, washed with 75% ethanol followed by centrifugation at 10,000 rpm, and allowed to air dry at room temperature. Finally, the dried RNA pellet was re-suspended in DEPC water (20 μl) and quantified in a nano-drop spectrophotometer (Thermo Scientific, USA). Approximately, 1 μg of cDNA was prepared from isolated RNA with a total volume of 11.5 μl (RNA + DEPC water). Next, oligo dT (1 μl) was added and incubated at 70 °C for 5 min inside the PCR machine (Applied Biosystems, USA). The mixture was taken out and placed in chilled ice for 5 min followed by the addition of PCR master mix. The mixture was given a short vortex to mix well followed by incubation at 37 °C for 5 min. Then, 1 μl of reverse transcriptase was added and incubated at 42 °C for 60 min inside the PCR machine. Finally, the mixture was again incubated at 70 °C for 10 min after that the mixture was taken out and kept on ice for 5–10 min and stored at −20 °C for further processing.

**Real-time PCR**

The gene expressions of the prepared cDNA samples were analyzed by real-time quantitative PCR analyses. The reactions were carried in 0.2 ml strip tubes/96-well plates (Applied Biosystems, USA) having a final reaction volume of 10 μl. The reaction mixture containing 10 ng cDNA, 2 ng of both forward and reverse primers along with 1×SYBR-Green PCR master mixture (Power SYBR-Green PCR Master Mix; Applied Biosystems, USA) was prepared. The quantitative real-time PCR was carried out using the RT-PCR system (Applied Biosystems, USA). All the reactions were performed in triplicates and the expression of enzyme GAPDH was used as a housekeeping gene for the following experimental studies. Primer sequences for spliced XBP-1 mRNA were forward 5′-CCCCAGAACAATCCTCC CAT-3′, reverse 5′-ACATGACTGGTGCCAGTGTG-3′, unspliced sequences were forward: 5′-AGACTGCTGCCCAT-3′, reverse 5′-AGTTGAGAATGGCCCA ACA-3′. Primer sequences for IRE1-α were forward 5′-CGG AACGTGATCCGCTACTTCT-3′ and reverse 5′-CGC AAAGCTCTTGCTGCCACA-3′ (IDT Inc., Singapore). The reaction mixtures were incubated at 95 °C for 2 min, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C, respectively (Ahmadiany et al. 2019). Relative expression of transcript levels of each sample was demonstrated according to the 2^ΔΔCT methods.

**Immunoblotting**

After washing twice with 1× PBS, cultured fd-FLS were lysed in cell lysis buffer (1×) contains PKC (Protein Kinase C). Quantification of total protein was done using Bradford reagent. Cell lysate proteins (50 μg) were separated by SDS–polyacrylamide gel electrophoresis, transferred into nitrocellulose membrane, and (3% BSA dissolved in Tris buffer saline or TBS) was used for blocking for 2 h at room temperature and incubated overnight at 4 °C with desired 10 antibodies (Dilution- 1:10,000). After washing twice with TBST (Tris Buffer solution with Tween), the membrane was incubated with HRP conjugated 2° antibody (Dilution- 1:500) for 2 h at room temperature. The membrane was washed four times with TBST followed by development via chemiluminescence using a gel documentation system (Bio-Rad-Chemidoc, XRS +), quantified by Image J, (Image J, 1.40, USA), and normalized by α-tubulin for further analysis. Selected immunoblots were stripped of antibodies with stripping buffer (62.5 mM Tris (pH 6.8), 2% SDS in 100 mM β-mercaptoethanol incubated at 50 °C for approximately 30–45 min) (Kanai et al. 1997).

**Caspase 3 assay**

Caspase-3 activity in synovial fibroblast cells was detected by using a caspase-3 assay kit ([BioVision, Sandiego, USA] (cat no: K106)]. Cells were seeded in a six-well plate at 1X10^4 cells/well, incubated overnight with a combination of MTX (125 nM) and TF3 (10 μM) for 24 h. After completing the treatment, cells were lysed using cell lysis buffer (cell signaling) and 2× reaction buffer was added to each sample with 5 μl of DVD-pNA substrate, and cells were incubated at 37 °C for 1–2 h, followed by measurement in a spectrophotometer at 400–405 nM.
**Immunofluorescence staining and confocal microscopy**

Synovial fibroblast cells were seeded (1 × 10⁶ cells/ml) in a confocal dish (SPL Life Sciences) and incubated overnight. After treatment with either rapamycin or MTX + TF3 and rapamycin (200 nM), cells were fixed with 4% v/v paraformaldehyde (in 1 × PBS) at room temperature, washed twice, permeabilized with 0.2% triton X-100 in 1 × PBS for 20 min at room temperature. Cells were then blocked with 2% BSA, washed, and incubated overnight with primary antibody LC3B (ab51520) (1:100 dilution) at 4 °C. Cells were treated with fluorophore-conjugated secondary antibody (Invitrogen) and Hoechst (for nuclear staining, Invitrogen) diluted in 1% BSA –1 × PBS and viewed under Olympus FV10i-LIV (Olympus, Japan) confocal laser-scanning microscope (magnification: 60×4/1.35).

**Statistical analysis**

Data were analyzed by unpaired *t* test or one way ANOVA for parametric data followed by post hoc Turkey’s multiple comparison. For nonparametric data Mann–Whitney or Kruskal–Wallis test was done followed by post hoc Dunn’s multiple comparison. Data were expressed as median (IQR) or mean ± SD. Correlation was done using Pearson’s correlation coefficient for parametric data and Spearman rank correlation coefficient for nonparametric data using Graph Pad Prism software, version 5.0 (Graph Pad Software Inc, La Jolla, CA, USA); *p* < 0.05 was considered as statistically significant.

**Results**

**Clinical characterization of RA and OA patients**

Patients with active RA (*n* = 11) and OA (*n* = 10) were enrolled in this study. Comparative analyses of laboratory markers such as CRP and anti-CCP were significantly higher in RA compared to OA (*p* < 0.001 and *p* < 0.001, respectively) in SF from knee joints suggesting inflammation was predominant in patients with RA (Table 1).

**fd-FLS exhibits an overexpressed level of inflammatory markers**

Immunophenotyping characterisation of *fd-FLS* was conducted by flow cytometric assay. Isolated *fd-FLS* (from SF) sourced from knee joints of RA and OA patients were stained with anti-human CD90 FITC. The result showed that the frequency of CD90⁺ population was higher in *fd-FLS* of RA (*p* < 0.01) than in OA [91.90 (88.16–95.73) %, vs 4.005 (2.423–8.925) %, Fig. 1A i, ii, iii, iv and B]. Inflammatory marker like CRP was significantly upregulated (31 folds) in *fd-FLS* (*p < 0.01*) of patients with RA compared to OA [57.80 (16.95–55.73) vs. 2.9 (1.42–8.92)%, Fig. 1C]. No significant correlation was found between CRP and CD90⁺ population in patients with OA (Fig. 1D, i), whereas the frequency significantly varied in patients with RA (*r* = 0.7, *p* = 0.007, Fig. 1D, ii).

Level of pro-inflammatory markers like TNF-α was significantly upregulated in RA compared to OA (*p < 0.001*) [856.0 (528.0–952.3) vs. 6.50 (3.88–16.35)%, Fig. 1E]. Significant correlation was found between CRP and CD90⁺ population in patients with OA (Fig. 1F, i) and with CD90⁺ population in patients with RA (*r* = 0.6, *p* = 0.03) and IL-6 (*r* = 0.6, *p* = 0.02) in patients with RA (Fig. 1E iv, vi), while OA patients did not correlate (Fig. 1E iii, v).

**Combination treatment of TF3 with MTX reduces the cell viability of *fd-FLS* sourced from SF of patients with RA**

The anti-proliferative effects of TF3 or MTX or in a combination of both on *fd-FLS* were determined by MTT assay. RA-FLS were treated with TF3 [0–50 μM, (5, 10, 15, 20, 30, 40, 50 μM)] for 18, 24 and 48 h. Cell viability was determined at IC₅₀ dose at respective time points. We found that TF3 administration at 20 μM dose for 24 h was most effective in reducing the cell viability compared to other time points (Fig. 2A). Further increasing the TF3 dose above 20 μM (4–6 days), no change was furnished in cell death.

### Table 1 Clinical data of RA (*n* = 11) and OA (*n* = 10) patients from synovial fluid

| Variables            | RA patients (N = 11) | OA (N = 10) | p value |
|----------------------|----------------------|-------------|---------|
| Age (years)          | 41.45 ± 11.84        | 43 ± 11.03  | 0.6     |
| Gender (M:F)         | 2/9                  | 1/9         |         |
| Disease duration (years) | 4.280 ± 2.821       | 5.34 ± 3.05 |         |
| Anti CCP (RU/ml)     | 242.8 ± 123.5        | 13.56 ± 5.67| <0.0016*|
| CRP (mg/l)           | 39.02 ± 31.06        | 2.833 ± 0.40| <0.001* |
| DAS 28-CRP           | 5.175 (4.303–5.715)  | –           | –       |

Baseline, demographic and clinical data of the patients group from synovial fluid. Data expressed as mean ± SD

DAS 28-CRP represents as median value (inter-quartile range)

Anti-CCP anti-citrullinated protein antibody; CRP C-reactive protein, DAS 28-CRP disease activity Score 28

* p value < 0.05 which is considered as significant

**Median value (inter-quartile range)
and saturation was reached at 48 h time point. MTX (1 μM) was able to diminish 50% (IC50) of cultured fd-FLS when incubated for 18 h and 24 h (Fig. 2B), (the result of finding at 18 h is given in supplementary Fig. 1). Since MTX is very efficient in low doses, so we fixed the IC25 dose of MTX and determined the optimum effective dose of TF3 in combination on fd-FLS isolated from RA for 24 h. Our investigation showed effective minimal IC50 concentration of TF3 was 10 μM over a fixed dose of MTX (125 nM, IC25) (Fig. 2C). Based on these findings, we chose TF3 (10 μM) in combination with MTX (125 nM) for our further studies.

Combination of MTX and TF3 downregulates the HIF-1α expression in fd-FLS from SF of RA patients

Inflammation associated with uncontrolled cell proliferation is one of the causes for hypoxic stress generation in cellular microenvironment (Qu et al. 2018), which is a regular phenomenon in RA. Hypoxic stress was investigated in RASF-derived fd-FLS after treatment with MTX and TF3. It was observed from immunoblot analysis that fd-FLS (without treatment) had a profound expression of HIF-1α enzyme suggesting that HIF-1α might be induced under chronic pro-inflammatory stress (Fig. 3A). Treatment with MTX in combination with TF3 (10 μM, 24 h) reduced the expression of HIF-1α significantly (p < 0.001) compared to their individual administration (p < 0.05 for MTX and TF3) in fd-FLS obtained from SF of RA (Fig. 3A, B).

Extensive expression of HIF-1α in inflammatory diseases triggers the downstream signaling molecules like VEGF and Ang-1 (Krock et al. 2011). As expected, VEGF level was found high in fd-FLS from RA patients than OA (p < 0.05), and combinatorial treatment (MTX and TF3) showed a significant decline in VEGF level (p < 0.001, Fig. 3C i). Similarly, the Ang-1 level was significantly (p < 0.05) higher in RA compared to OA, which was drastically declined upon dual administration of MTX and TF3 in fd-FLS (p < 0.01, Fig. 3C ii). Collectively, these data suggested that MTX and TF3 in combination might reduce the angiogenic potency of fd-FLS in RA patients.
Fig. 2 Cell viability assay after treatment with MTX and TF3 to fd-FLS from RA patients by MTT assay. A The synergistic inhibitory effect of TF3 on cells was determined by MTT assay. Briefly, cultured cells (5 × 10⁴) were treated with TF3 (0-50 µM) for a period of (18, 24, 48 h). B Effect of MTX on fd-FLS from RA patients. 5 × 10⁴ cells/well was seeded on 96 well plates and were incubated for 24 h after treatment with MTX (0.2-1 µM). C Effect of TF3 (5-30 µM) over a fixed dose of MTX (IC₅₀ = 125 nM) was studied in fd-FLS after incubation for 24 h followed by MTT assay.

Fig. 3 Determination of (HIF-1α) expression in fd-FLS after treatment with TF3 alone or in combination with MTX. A Interpretation of HIF-1α expression by immunoblot assay. B Different experimental sets by densitometric scans of HIF-1α immunoblot. C Determination of angiogenic markers. (i) Expression of VEGF, ***p < 0.001 compared to untreated group vs MTX (125 nM), TF3 (10 µM), MTX + TF3 (125 nM + 10 µM). (ii) Expression of ANG-1, **p < 0.01 compared to untreated group vs MTX (125 nM), TF3 (10 µM), MTX + TF3 (125 nM + 10 µM); Unt untreated.
'Add on' effect of TF3 with MTX upon hypoxia-induced ER stress in fd-FLS from RA patients

Earlier reports suggest that inflammation-mediated hypoxic stress within fibroblasts is often associated with the development of UPR and ER stress (Park et al. 2014). To address this, well-established ER stress markers (GRP78 and HSP70) were analyzed in the fd-FLS of different studied groups. Our immunoblot data demonstrated overexpression of GRP78, in fd-FLS sourced from patients with RA compared to OA (Fig. 4A), as represented in density scan data \( p < 0.05 \) (Fig. 4B i). Individual administration of MTX or TF3 was noticeably less effective in reducing the expression of GRP78 and consequently, hypoxia-mediated ER stress compared to combinatorial administration which efficiently hindered (~ 5-fold) the expression of GRP78 in fd-FLS of RA than OA. Likewise, HSP70 (a molecular chaperone), an established intracellular marker of ER stress associated in RA pathogenesis (Luo et al. 2008), was also assessed in our study. Data showed the expression of HSP70 followed a similar trend as observed in GRP78 in fd-FLS (3.43-fold increase) of RA compared to OA, \( p < 0.05 \) (Fig. 4B ii). MTX + TF3 efficiently reduced the HSP70 expression in fd-FLS from RA than their individual application.

Under UPR stress, the downstream transduction of signaling milieu from GRP78 is emanated primarily in three directions (Wang et al. 2009). One of which is IRE1α, which receives the pro-apoptotic signal. ER stress causes oligomerization and activation of the kinase domain in IRE1α (Junjappa et al. 2018). Activation of kinase domain-associated RNase domain in IRE1α splices X-binding protein 1 (XBP1) mRNA. This spliced mRNA in turn regulates ER-associated genes especially apoptosis (Wu et al. 2015). In our study, IRE1α was significantly upregulated in fd-FLS of RA, whereas combined treatment with TF3 and MTX markedly reduced the expression much closer to control cells (OA) (Fig. 4C i). Similarly, the mRNA level of spliced XBP-1 was quite overexpressed in fd-FLS from RA patients than OA, and after administration of MTX + TF3 (Fig. 4C ii), the expression level of spliced XBP-1 was drastically declined close to fd-FLS from OA than their individual treatments. It is already known that spliced XBP-1 mRNA acts as a transcription regulator and modulate expressions of various pro and anti-apoptotic genes. One of the prominent genes is pro-apoptotic protein CCAAT enhancer-binding protein homologous protein (CHOP) which is activated by spliced XBP-1 to initiates the apoptotic process (Yang et al. 2017). Immunoblot analysis of CHOP showed that it was down-regulated in fd-FLS derived from RA compared to OA patients. Combinatorial administration of MTX and TF3 significantly \( p < 0.001 \) enhanced the CHOP expression (fivefold) compared to their individual application \( p < 0.001 \),

![Fig. 4 Determination of hypoxia-mediated ER stress in fd-FLS from RA patients after combination treatment with TF3 and MTX.](image-url)

- A: Expressions of GRP78 and hsp70 were determined by the immunoblot technique. Data were normalized by α-tubulin. Immuno-reactive bands were identified by densitometric scans in different studied groups. (i) GRP78 expression, \* \( p < 0.05 \) compared to untreated group vs MTX (125 nM), TF3 (10 µM), MTX + TF3(125 nM + 10 µM), (ii) HSP70 expression, \* \( p < 0.05 \) compared to untreated group vs MTX (125 nM), TF3 (10 µM), MTX + TF3(125 nM + 10 µM).
- B: Gene expression level of IRE-1α, (i) sXBP-1. (i) CHOP expression determined by immunoblot assay. (ii) Densitometry scan of CHOP in the different studied groups. *** \( p < 0.001 \) compared to untreated group vs MTX (125 nM), TF3 (10 µM), MTX + TF3(125 nM + 10 µM); Unt untreated.
Combination therapy of TF3 with MTX induces apoptosis in fd-FLS derived from RA patients by inducing BAX and suppressing Bcl-2

fd-FLS from RA patients exhibit resistance to ER stress-mediated cell death (Park et al. 2014; Ahmadian et al. 2019). Our findings showed that in RA fd-FLS, CHOP was down-regulated which might modulate the status of apoptosis. Since it is known that both anti-apoptotic proteins (Bcl-2) and pro-apoptotic Bax (Bcl-2-associated X protein) are targeted by CHOP (Hu et al. 2018), therefore, the status of these proteins were investigated. Immunoblot data showed that Bcl-2 expression was concomitantly decreased after application of individual drug therapy in fd-FLS from RA (Fig. 5A, B i). The most significant decrease in expression was observed after combination treatment with MTX and TF3. On the other hand, the expression of Bax was found to be low in untreated fd-FLS cells but combination therapy with MTX and TF3 reversed the expression significantly thereby indicating initiation of the pro-apoptotic signals (Fig. 5A, B ii).

Next, we examined the impact of Bax on programmed cell death and its consequences after administration of combination therapy with MTX and TF3 in fd-FLS from RA patients. Findings showed that the frequency of Annexin V+ apoptotic cells was 5.450 (4.030–5.640) in untreated fd-FLS of RA patients. Administration of MTX (125 nM) and TF3 (5 μM) in combination increased the frequency of apoptotic cells [15.37 (14.56–16.50)] compared to their individual application which was further extrapolated from the percentage of apoptosis (Fig. 5C i, ii). MTX + TF3 treatment on caspases (initiator and executioner caspases) was also determined. Immunoblot study showed that the expressions of both initiators (caspase 9) as well as executioner (caspase 3) (total and cleaved) were down-regulated in fd-FLS of RA compared to OA (Fig. 5D ii, i). Both cleaved caspase 3 and caspase 9 expressions were increased significantly (fivefold and sixfold, respectively) after dual administration of MTX with TF3 (p < 0.05) in fd-FLS from RA than untreated fd-FLS (Fig. 5D iii, iv). The activity of caspase 3 was also monitored by a colorimetric assay. MTX + TF3 treatment restored the activity of executioner caspase in fd-FLS from RA than untreated cells, (p < 0.01) (Fig. 5E).

MTX and TF3 administration synergistically inhibit ER stress-induced autophagy in fd-FLS derived from RA patients

In RA fd-FLS, under ER stress, a halt in the apoptotic pathway is often linked with activation of autophagosomal proteins leading to prolonged cell survival (Vomero et al. 2018). Dissociation of anti-apoptotic Bcl-2 from Beclin-1 (known to be a prime regulatory molecule in the autophagy startup process) induces autophagy (Decuyper et al. 2012). To evaluate the expression of Beclin-1 in the modulation of the apoptotic pathway, cells were treated with MTX and TF3 and quantified by immunoblot assay. The image showed that Beclin-1 in untreated RA fd-FLS was highly expressed than cells from OA patients (Fig. 6A, B). Individual treatment with MTX or TF3 was not sufficient enough in suppressing the expression of Beclin-1 in fd-FLS compared to their combined application which was quite evident from their densitometric scans. The densitometric ratio between Beclin-1 and Bcl-2 was measured to assess the switching over between apoptotic and autophagic pathway. Data suggested a significant decrease in beclin: Bcl-2 ratio in fd-FLS from RA indicating a shift to the autophagic pathway. MTX+TF3 treatment to RA fd-FLS reversed the shifting towards programmed cell death pathway significantly compared to the individual drug administration.

Expressions of autophagic inducer proteins (LC3B and p62) were examined in fd-FLS obtained from RA and OA. Data showed that both LC3B and p62 were highly expressed in untreated fd-FLS of RA compared to OA (Fig. 6A, C, D). Treatment with MTX or TF3 alone was less effective in reducing the expression of both of these proteins compared to the combination therapy, and it was further supported by the relative band intensities data (Fig. 6A–D). These findings suggested that TF3 in combination with MTX might play a crucial role in downregulating the expression of Beclin-1, p62, LC3B thereby might halt the entry to the autophagy pathway. Further analysis was done to evaluate the mode of action of LC3B protein. It is known that migration of this protein to the nucleus is necessary to trigger autophagic activity (Huang and Liu 2015). Confocal micrographs showed localization of LC3B within the nucleus (red dots) of fd-FLS from RA patients (Fig. 6E). Individual MTX and TF3 treatment resulted in an insignificant reduction in the accumulation of LC3B within the nucleus (data not shown). Rapamycin, one of the inducers of autophagy, was administered which significantly increased autophagic puncta (Andersson et al. 2016), whereas combination treatment with rapamycin depicted lesser accumulation of LC3BI protein within the nucleus of fd-FLS from RA suggesting a reduction in autophagic activity.

MTX + TF3 treatment halt the autophagy and induce apoptosis in fd-FLS from RA

This finding instigated us to see whether inhibition in autophagic proteins could divert the cellular physiology to apoptotic death or not. To investigate this, fd-FLS of both RA and OA were treated with autophagic blocker 3-MA, and
apo-flow cytometry and Western blot analysis were performed to determine the expression of pro-apoptotic and anti-apoptotic proteins. The results showed that the expression of pro-apoptotic protein Bax was upregulated, while the expression of anti-apoptotic protein Bcl2 was downregulated in the treated group compared to the control group. This indicates that the treatment with MTX and/or TF3 induces apoptosis in fd-FLS from RA patients.

MTX + TF3 administration induced apoptosis in fd-FLS from RA patients. A Representation of immunoblot data of Bcl2 and Bax after treatment with TF3, MTX, and in combination. B Densitometry scan of (i) Bcl2 (ii) Bax. C (i) Cell death was measured by FACS analysis using Annexin V-FITC in fd-FLS after treatment with TF3(10 µM), MTX(125 nM) and in combination MTX + TF3(125 nM + 10 µM). (ii) Percentage of Annexin V positive cells were represented in the histogram. D Immunoblot data after treatment with TF3(10 µM), MTX (125 nM) and in combination MTX + TF3(125 nM + 10 µM). (i) Determination of caspase 3 and cleaved caspase 3, (ii) Expression of caspase 9 and cleaved caspase 9. Densitometry scans of (iii) Caspase-3 (iv) Caspase 9 in different studied groups. E Estimation of caspase 3 activity by colorimetric study in fd-FLS from RA after different drug administration; TF3(10 µM), MTX(125 nM) and in combination MTX + TF3(125 nM + 10 µM). Data represent as mean ± SD (*p < 0.05, **p < 0.01, ***p < 0.001); Unt untreated.

MTX + TF3(125 nM + 10 µM). (i) Determination of caspase 3 and cleaved caspase 3, (ii) Expression of caspase 9 and cleaved caspase 9. Densitometry scans of (iii) Caspase-3 (iv) Caspase 9 in different studied groups. E Estimation of caspase 3 activity by colorimetric study in fd-FLS from RA after different drug administration; TF3(10 µM), MTX(125 nM) and in combination MTX + TF3(125 nM + 10 µM). Data represent as mean ± SD (*p < 0.05, **p < 0.01, ***p < 0.001); Unt untreated.
was demonstrated when 3-MA was administration with TF3 + MTX in *fd-FLS* from RA [31.01 (24.50–38.310)] (Fig. 7A, B).

**Discussion**

Cell proliferation and resistance towards apoptosis are some of the hallmark incidences that take place in the RA synovium (Nygaard and Firestein 2020). Autophagy is known to be a protective mechanism against the pro-apoptotic signal (Heymann 2006), though many have shown its association in inducing programmed cell death especially in cancer cells and other inflammatory diseases (Jung et al. 2020). In RA, the *fd-FLS* are known to evade apoptotic signals by inducing autophagy (Xu et al. 2015) thereby continue their survival and pannus expansion (Zhu et al. 2017). Prolonged accumulation of inflammatory loads due to infiltration of pro-inflammatory cytokines and concomitant building up of hypoxic stress promote two vital incidents. One of which is the release of angiogenic factors due to hypoxia and the onset of intracellular stress accumulation. ER stress is one such incident that takes place in the RA synovium, in which cellular protein folding machineries are destabilized secondarily to the hypoxic and inflammatory loads.

Among the conventional medications, MTX has always remained a first-line defense against RA (Pincus et al. 2003). Previous studies showed that prolonged administration of the conventional synthetic DMARDs (Methotrexate, leflunomide, sulfasalazine) can cause various systemic side effects like liver toxicity, etc. (Wang et al. 2018). In RA, the anti-inflammatory effect of MTX is chiefly exerted by adenosine release. Originally, MTX acts as a folate inhibitor by its antagonistic effect on dihydrofolate reductase enzyme. This agent is recommended for the treatment of leukemia at a very high dose (Sakura et al. 2018). Inhibition of purine metabolism by halting the cell cycle movement at the S phase causing apoptosis is another mechanism of action of this agent (Goncharova and Frankfurt 1976; Fairbanks et al. 1999). However, MTX shows its efficacy at a very low dose in RA (Steinsson et al. 1982). In RA, it is highly elusive to ascertain that whether a low dose of MTX arrests the cell cycle and initiates caspases activities or not. On the other hand, a higher dose of MTX is not recommended to administer in RA patients due to its severe complications (Bello et al. 2017). Considering these issues, MTX (low dose) has been chosen as a primary drug and decided to co-administer with a compound for its low side effect profile, and effective in inducing pro-caspases activities.

Polyphenolic derivatives from black tea have therapeutic properties against several human disorders ranging from metabolic to cardiovascular and cancer (Khan and Mukhtar 2013). The mode of actions of these therapeutics generally ranges from anti-oxidative, anti-inflammatory to pro-apoptotic effects (Gosslau et al. 2018). TF3, an active compound of black tea exhibits the strongest anti-inflammatory, anti-angiogenic and pro-apoptotic properties in different cell types of various disease models (Gao et al. 2016). Thus, the study was conducted to investigate the efficacy of polyphenolic derivative of black tea (TF3) in combination with MTX (low dose) in modulating the angiogenesis and autophagy-mediated cell proliferation in *fd-FLS* from RA patients. It is well evident from other studies and ours as well that levels of pro-inflammatory cytokines (TNF-α, IL-6) are highly expressed in synovial fluid of RA patients than OA. Our findings also revealed that increased expressions of pro-inflammatory markers and CRP levels have a positive association with an increased *fd-FLS* expression in RA synovial fluid than OA. A substantial increase in the *fd-FLS* population within synovial joints might reflect the persistence of inflammation which has been identified in other studies (Alunno et al. 2017). Our finding also showed that administration of a low dose of MTX in combination with TF3 was more efficient in killing the cells (*fd-FLS*) and downregulating the inflammatory loads compared to individual administration (TF3/MTX). This observation is well consistent with the anti-proliferative, anti-inflammatory, and pro-apoptotic action of TF3 on inflammatory and tumor cells (Tu et al. 2016). The proliferation of synovial fibroblasts in RA is often associated with the building up of hypoxic stress (Quinonez-Flores et al. 2016), which results in release of angiogenic factors within the synovium (Elshabrawy et al. 2015). HIF-1α and several other cytokines like TNF-α, IL-1β instigate *fd-FLS* to release VEGF and Ang-1 in synovial joints (MacDonald et al. 2018) which was evident from our observations. Therefore, the RA *fd-FLS* has greater scope to be stimulated by these molecules than in OA. TF3 has potent anti-angiogenic action on several cell types, especially on tumor cells where it hinders the expressions of hypoxic and angiogenic factors (Gao et al. 2016). Notably, we also found a significant downregulation of HIF-1α, VEGF, and Ang-1 factors in *fd-FLS* from RA after TF3 + MTX treatment.

The pro-inflammatory cytokines and hypoxic stress in *fd-FLS* trigger downstream UPR and subsequent ER stress which play a pivotal role in disease pathogenesis and progression in RA (Park et al. 2014). Within the hypoxic microenvironment of RA synovium, proliferative *fd-FLSs* are induced to accumulate misfolded proteins, which in turn cause UPR and upregulation of ER stress factors like GRP78, HSP70, and CHOP (Yoo et al. 2012). GRP78 is a molecular chaperon that exhibits anti-apoptotic properties against numerous inflammatory, tumourigenic and rheumatic diseases and serves as a pivotal factor in RA pathogenesis (Yoo et al. 2012). Significant elevation of the GRP78 level was observed in the RA *fd-FLS*, but not in OA *fd-FLS*. GRP78 in *fd-FLS* could serve as a therapeutic target (Park...
et al. 2014). HSP70 is found to be elevated in RA fd-FLS which is well consistent with earlier observations (Kang et al. 2009). The level of HSP70 was reduced after TF3 alone or TF3 + MTX combination. These two findings indicate the efficacy of combination therapy in reducing the expression of ER sensor proteins and ER stress response.

During ER stress, the activated GRP78 conveys a signal downstream to a crucial molecule IRE1α, that holds the key for enzymatic activity leading to cell survival or death (Ahmadiany et al. 2019) via another regulatory molecule XBP-1 mRNA. The spliced XBP-1 mRNA is responsible for inducing apoptotic signals (Zeng et al. 2009). Our results showed that the expressions of IRE1α and XBP-1 were elevated in fd-FLS of RA patients as observed by others also (Kabala et al. 2017). One earlier study shows that an elevated XBP-1 level contributes to an apoptosis-resistant phenotype in RA fd-FLS (Ahmadiany et al. 2019). In our work, the levels of both the IRE1α and sXBP-1 were reduced after MTX + TF3 application thereby highlighting a probable pro-apoptotic activity of TF3. The pro-apoptotic effect of the TF3 + MTX combination was further confirmed by monitoring another downstream sensor protein CHOP which is responsible for caspases activation. In accordance with previous studies, we also found a lower expression of CHOP in RA fd-FLS compared to OA, which further justifies apoptosis-resistant characteristics in RA fd-FLS (Bustamante et al. 2017).

Since CHOP is a transcription factor, it is known to regulate numerous anti-apoptotic factors especially Bcl-2 and pro-apoptotic like Bax (Hu et al. 2018). Overexpressed BCL-2 and under-expressed Bax were found in fd-FLS from RA implicating the importance of CHOP. On the other hand, TF3 or TF3 + MTX downregulated the expressions of Bax and Bcl-2 through upregulating CHOP which can be predicted as switching from anti to pro-apoptotic phenotype in RA fd-FLS. This chemo-therapeutic activity of TF3 by maneuvering Bcl-2/Bax has previously been argued in cancer and inflammatory cells (Leone et al. 2003). Modulation of caspases (both initiator and executioner) activities in RA fd-FLS is a well-known mechanism by which apoptosis is halted and localized cellular proliferation continues (Smith and Walker 2004). TF3 + MTX induced caspase activities and apoptosis in fd-FLS as observed in our findings. Previously, TF3 has been shown to promote apoptotic genes in combination with phytochemicals or other drugs in various cancer and inflammatory cells (Pan et al. 2018) which is consistent with our findings.

Inhibition in the natural apoptotic pathway in RA fd-FLS instigated us to speculate the role of autophagy in their survival. Recently, it has been shown that, autophagy acts as a protective mechanism against apoptosis in RA fd-FLS, and knocking down the autophagic genes promotes apoptosis (Vomero et al. 2018). Beclin-1 is one such factor whose modulation in RA fd-FLS causes persistence of autophagy-mediated survival (Xu et al. 2015) as observed from our immunoblot analysis. Another important factor, LC3B works downstream of Beclin-1 and regulates the autophagic process where LC3B-I converts to LC3B-II after accumulation in autophagosome (Vomero et al. 2018). LC3B-II thereafter becomes the structural entity of autophagosomal vesicle. In our study, elevated levels of LC3B-II: LC3B-I were observed in RA fd-FLS, indicating an augmented autophagy pathway in RA. These observations were similar to the findings of a previous study (Zhu et al. 2017). p62 is an adapter protein whose binding is necessary for the transformation of LC3B-I– LC3B-II and subsequent formation of autophagosomal bodies (Runwal et al. 2019). Higher expression of p62 in our data indicated autophagosomal vesicles were formed in RA fd-FLS. Individual MTX or TF3 or combined treatment showed their ability to reduce autophagic proteins like LC3B-I, LC3B-II, and p62. Many researchers observed that isolated TF3 administration can induce autophagic flux by upregulating LC3B-II and Beclin-1 levels in different cell types and cause cell death which contradicted our findings (Shen et al. 2019). Although these findings only suggest that autophagy has a role in promoting cell death, in RA fd-FLS, apoptosis is restricted by autophagy. TF3 + MTX is effective in inducing apoptosis by halting autophagy. This phenomenon was further justified by applying autophagic inducer (rapamycin) to fd-FLS along with TF3 and MTX to show their involvement in restricting autophagy. These molecules proved to be successful in reducing the level of autophagosomal proteins LC3B-II accumulation as confirmed by confocal imaging. This may be predicted as the modulation of the functionality of Beclin-1 and p62 under combination therapy.

Induction of apoptosis by blocking autophagy in fd-FLS from RA in our treatment was confirmed using autophagy blocker 3MA. MTX and TF3 combination showed their ability to promote apoptosis in 3MA-mediated autophagy restricted fd-FLS from RA, which confirms their apoptosis...
induction ability by restricting autophagy-mediated cell survivability in fd-FLS.

In this ex vivo study, the combination treatment of MTX + TF3 showed a synergistic effect in reversing the imbalanced autophagy and apoptosis pathway in the fd FLS of RA patients but not in OA patients. The next pragmatic approach would be to observe the effect of the combination in the animal model of RA and to assess the efficacy in patients with RA.

**Conclusion**

Overexpression of the pro-inflammatory cytokines and hypoxic stress induces the pathological alterations in RA fd-FLS, leading to angiogenesis, cell proliferation, and dysregulated cell survival by modulating autophagy and apoptosis regulatory proteins. Co-administration of TF3 with MTX downregulated the angiogenic and hypoxic markers (HIF1-α, VEGF, and ANG-1) in RA fd-FLS, consequently countering the hypoxia-mediated ER-stress response and its imbalanced downstream pathway. Upregulation of ER stress-induced autophagy and downregulation of apoptosis in RA fd -FLS was significantly halted by the MTX and TF3 combination treatment, as evident from the pre- and post-treatment assessment of the respective markers, and finally supported by 3-MA administration with TF3 or MTX in fd-FLS. From the pathologic viewpoint, disruption of the abnormal autophagy and apoptotic pathway in RA fd-FLS by MTX and TF3 combination is an important finding, as it may provide additional benefit to MTX monotherapy in limiting the synovial inflammation in RA. Further studies in this aspect are required to derive a definite conclusion.

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Author contributions SM, AS, SM, PSM, AG, PC and MC initiated the concept and designed the experiments. SM, AB, DB, SC, SC, AS performed the experiments. SN assisted in confocal experiments. SM, AS, SM, AB, PSM and AG wrote the manuscript. AC extracted the synovial fluid from patients and assisted in manuscript preparation.

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Code availability Not applicable.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethics approval This study was approved by the Institutional Ethics Committee (No: Inst/IEC/24.02.2014) dated 24th February, 2014.

Consent to participate This study was conducted using the biological samples collected from selected patients visited our OPD. For every patient, prior written consent was taken before enrolling him/her in the study.

Consent to publish Not applicable.

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