The Effects of Atorvastatin and Methotrexate on Freund's Adjuvant–induced Arthritis in Rats

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Abstract

Background: Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis that progressively destroys synovial joints. Statins have been shown to have anti-inflammatory and/or immunomodulatory activities, many studies have focused on the ability of statins to modulate chronic inflammatory diseases, such as RA. Methotrexate (MTX) has become the leading disease modifying antirheumatic drugs (DMARD) for the treatment of RA and other autoimmune diseases. Aim: to determine the anti-inflammatory effect of atorvastatin in combination with MTX on the course of experimentally -induced arthritis in rats. Materials and Methods: rats were divided into 6 groups(Control, arthritic control, atorvastatin, MTX, combination of both in full and half doses) for 28 days. Afterwards they were sacrificed and serum level of TNF-α was determined together with the histopathology for the joints of each group. Results: The oral administration of atorvastatin (5-10mg /kg/day) and MTX (0.3-0.5 mg/kg/day) in rats that were previously injected with CFA (either as a monotherapy with atorvastatin or MTX or as a combination of both in full or half doses for 28 days) significantly lowered TNF-α levels in sera. They also significantly reduced the histopathological changes compared with the arthritic control group. The best results were for the combination treatment especially with the full dose regimen. Conclusion: Statins possess a useful anti inflammatory effect in patients of RA especially when combined with other DMARDs.

Keywords: tumor necrosis factor-alpha, statins

Introduction

RA is a chronic inflammatory disorder, characterized by joint inflammation with concomitant destruction of cartilage and bone[1]. The pro-inflammatory cytokines; tumor necrosis factor-alpha (TNF-α), inter-leukin-1 (IL-1) and IL-6 are shown to play an important role in the pathophysiology of arthritis development in animal models and in human[2]. Statins represent a class of drugs that are widely prescribed for the treatment of hypercholesterolemia[3]. Statins influence multiple steps in the inflamm-atory process, including leukocyte migration and adhesion, T-cell stimulation, nitric oxide (NO) bioavailability, generation of free radicals, and angiogenesis[4]. Atorvastatin activates nuclear receptor Peroxisome proliferator-activated receptor gamma (PPAR-γ) in primary human monocytes in culture, in turn decreasing TNF-α production[5]. MTX has become the leading DMARD for the treatment of RA in much smaller doses than those used in the treatment of neoplasia[6]. Apart from the interference with folate biosynthesis, MTX was found to influence adenosine level and

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interact with adenosine receptors on immune-effector leucocytes[7] The use of MTX has been limited by some of its toxic manifestations[8]. The use of combination therapy of MTX or other DMARDs for RA has been suggested to improve efficacy and minimize toxic side effects via decreasing the dose of individual agents[8].

Materials and Methods

Animals
A total of 36 adult male albino Wistar rats, weighting 200–250 gm each, were used in this study. Animals were purchased from the national centre of research, Cairo, Egypt. Animals were housed in polyethylene cages at room temperature (under controlled environmental conditions) and were kept with free access to standard rodent chow diet and tap water ad libitum. Animals were allowed for acclimatization for one week before the start of the study.

Materials
Atorvastatin was purchased from Amoun Pharmaceutical Company, Cairo, Egypt as a white powder dissolved in distilled water. MTX was purchased from T3A Pharmaceutical Company, Cairo, Egypt as yellow powder dissolved in distilled water. CFA was purchased from Sigma biosciences, Egypt as a ten ml amber viscous liquid containing light-colored particulates. Each milliliter contains 1 mg mycobacterium tuberculosis; heat killed and dried 0.85 ml mineral oil and 0.15 ml mannide monooleate, stored at 2-8°C and shaken well before use. Rat TNF- α enzyme linked immunosorbent assay (ELISA) kits were purchased from Biosource Company, Camarillo, CA, USA.

Experimental groups
Animals were randomly allocated into six groups (6 animals each); they received the drugs by oral gavage in a volume of 2 ml/kg

for 28 days. *Group 1* (Normal control): non-arthritic that received distilled water (the drug vehicle) once daily. *Group 2* (Arthritic control): injected intradermally in the plantar surface of the right hind paw with 0.1 ml of CFA to induce arthritis and left for 10 days until the first signs of joint inflammation were noted. They received the drug vehicle once daily. *Group 3* (Atorvastatin group): Arthritic group treated with atorvastatin 10 mg /kg once daily[9]. *Group 4* (Methotrexate group): Arthritic group treated with methotrexate 0.5mg/kg twice a week[10]. *Group 5* (Atorvastatin plus Methotrexate group, full dose): Arthritic group treated with atorvastatin 10 mg /kg plus methotrexate 0.5mg/kg. *Group 6* (Atorvastatin plus Methotrexate group, half dose): Arthritic group treated with atorvastatin 5mg/kg plus methotrexate 0.3mg/kg. After 28 days, blood samples were collected from retro-orbital plexus of each rat before sacrificing them, via a microhematocrite capillary tube inserted at the inner canthus of the eye, 5 ml of blood was collected from each rat in a clean sterile test tube, centrifuged at 2000 ×g for 15 min after 30 min of collection Then, serum samples were withdrawn and stored at –80°C until cytokine estimation.

TNF-α measurement
Serum levels of TNF-α were measured by ELISA (Enzyme-linked immunosorbent assay) using the rat TNF-α kits according to manufacturer's instructions.

Histopathological examination:
After scarifying the animals by decapitation, each rat was fixed in a dissector plate, the right hind paw joint was cut about 0.5 cm above and below the joint. All the skin and muscles were trimmed away so that the joint was left with intact synovial membrane. The joint were decalcified by immersion in 3% nitric acid solution and main-

tained at room temperature for an average of 5-7 days. The acid solution was changed every 24 hours; decalcification was monitored by gently feeling the consistency of the bone by the tips of the fingers until it becomes firm to soft. Then it was fixed in 10% neutral buffered formalin for 2 days. The decalcified specimens were submitted to histologic processing which comprised sequential immersions in alcohol, xylne, liquid paraffin and lastly embedded in paraffin[11]. Serial sections were cut (3–4 mm thick), to be subjected to staining with hematoxylin and eosin (H&E) and examined microscopically for the presence of arthritis (concerning inflammation). Joints were graded using a modified version of a previously adoped system[12]. Cell infiltration in the synovial fluid was graded from 0 to 3 as follows: 0 (no)= no inflammatory cells in the joint cavity, 1 (mild)= a few inflammatory cells in the joint cavity, 2 (moderate)= joint cavity partly filled with inflammatory calls, 3 (marked)= joint cavity totally filled with inflammatory calls. Destruction of the cartilage was graded as follows: 0 (no)= normal appearance, 1 (mild)= minor signs of destruction, 2 (moderate)= up to 30% of destruction, 3 (marked)= more than 30% of destruction. Scoring was carried out by a blinded pathologist for the experimental groups.

Statistical analysis

All data were expressed as mean ± SEM. and analyzed using the Statistical Package of Social Sciences (SPSS) program version 17, (Chicago, IL, USA). All the comparisons among groups were carried out using one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test to assess the significance of the difference of mean values among groups. A value of $p < 0.05$ was considered to be statistically significant.
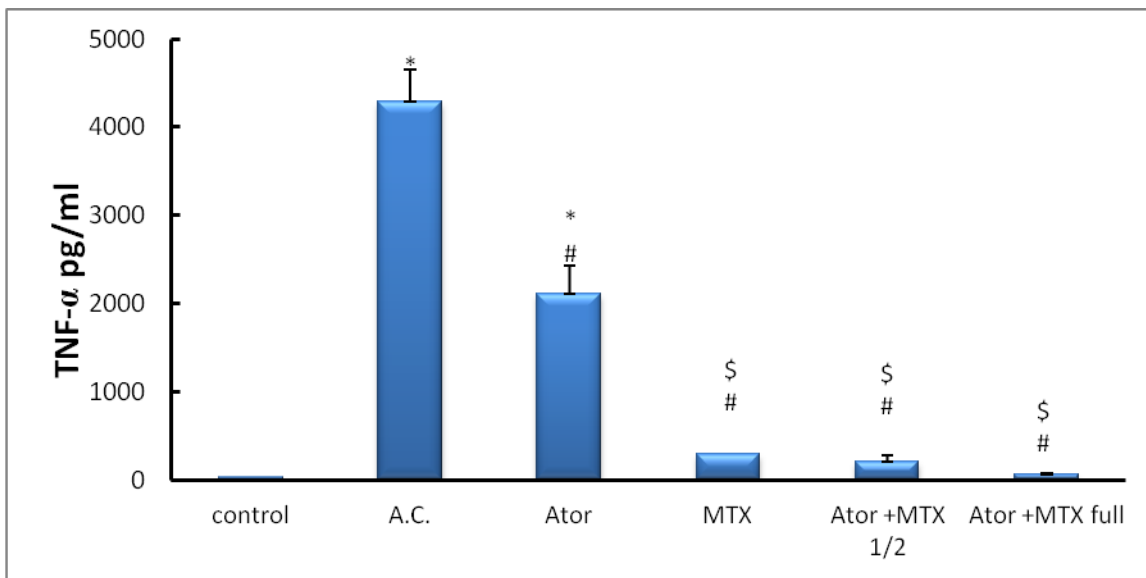

Figure 1: Serum TNF-α level in the experimental groups. Monotherapy with methotrexate or its combination with atorvastatin in both doses significantly ameliorated serum TNF-α level as compared to arthritic controls and atorvastatin treated groups. AC: arthritic control, Ator: atorvastatin, MTX: methotrexate. Results were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's test. * $P < 0.05$ *vs.* control group, # $P < 0.05$ *vs.* AC group, $ $P < 0.05$ *vs.* atorvastatin group, *n = 6.*

Results

Serum TNF-α level in groups under investigation

The results of the present study showed that CFA-induced arthritis in rats resulted in a significant increase in TNF-α serum level as compared to control rats ($p < 0.05$). Oral monotherapy with atorvastatin 10mg/kg/d or MTX 0.5 mg/kg twice a week for 28 days as well as combination of both in full or half doses resulted in a significant improvement of TNF-α serum level compared to arthritic control group best for full dose($P<0.05$, Fig. 1).

Histopathological changes:

Normal control group showed normal articular surface without edema and normal ligamentous parts as well as no inflammatory infiltration and no cartilage erosion was observed (Fig. 2). Joint sections in adjuvant arthritic control group showed marked thickening of the fibrous ligamentous parts, marked thickening of articular cartilage, marked lymphoplasmacytic inflammatory infiltrate and moderate neutrophilic infiltration about 50% associated with moderate degree of Fibrosis and mild cartilage destruction (Fig. 3). Atorvastatin monotherapy showed mild inflammatory infiltrate, moderate fibrosis, marked cartilaginous plate thickening, mild neutrophilic infiltration 5% and mild destruction of cartilage (Fig. 4). MTX monotherapy showed mild inflammation, moderate fibrosis, marked cartilaginous plate thickening and mild neutrophil infiltration 5% mild cartilage destruction (Fig. 5). Joint sections of combination (full dose) group showed Moderate inflammation, mild degree of fibrosis and neutrophil infiltration 20% (Fig.6) and no cartilage destruction. Those treated with both drugs (half dose) group showed moderate inflammation, mild degree of fibrosis, mild cartilaginous hyperplasia and neutrophil infiltration 10% and no cartilage

destruction (Fig. 7). The mean percent of neutrophil infiltration in the arthritic control group was 50% ± 1.87. Monotherapy with atorvastatin/MTX or their combination in the above mentioned doses resulted in a significant decrease in the percent of neutrophil infiltration as compared to arthritic control group (P<0.05). The combination treatment in either full or half doses resulted in a significant increase in neutrophil influx as compared to monotherapy with MTX or with atorvastatin (P<0.05, Fig. 8). The mean percent of neutrophil infiltration in the arthritic control group was 50% ± 1.87. Monotherapy or their combination in the above mentioned doses resulted in a significant decrease in the percent of neutrophil infiltration as compared to arthritic control group (P<0.05). The combination treatment in either full or half doses resulted in a significant increase in neutrophil influx as compared to monotherapy with methotrexate or with atorvastatin (P<0.05, Fig. 8). The mean score of cartilage destruction was 2.8±0.11 in the arthritic control group. Monotherapy with atorvastatin did not significantly decrease the cartilaginous plate thickening score whereas monotherapy with MTX as well as its combination with atorvastatin in half or full dose resulted in a significant decrease in the cartilage destruction score compared to arthritic control and atorvastatin groups. In addition, combination therapy in full doses significantly ameliorated this score as compared to monotherapy with methotrexate ($p<0.05$, Fig. 9).

Discussion

RA is a chronic inflammatory polyarthritis that progressively destroys synovial joints. There is no known cure and, despite improved therapy, the long-term prognosis remains poor, with a reduced average life expectancy[13]. Growing evidence suggests

that early treatment of RA with a combination of DMARDs may induce long-term and sustained functional and clinical outcomes in RA patients[14].
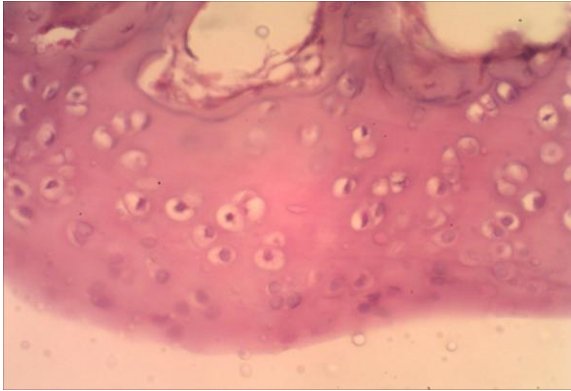

Figure 2: Right hind paw joint of normal control group. Normal joint space and cartilage with no inflammatory infiltrate. The arrows show normal joint cartilage. X 400
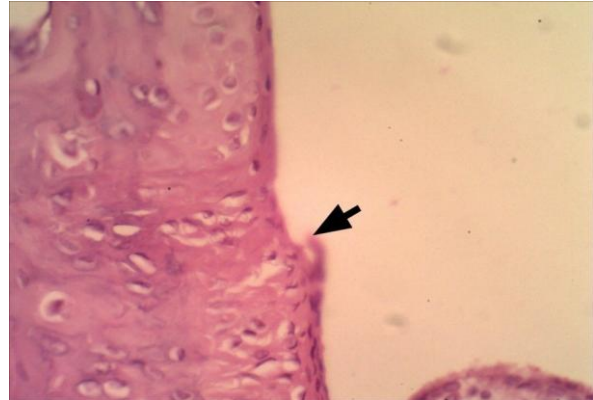

Figure 3: Right hind paw joint of arthritic control group. X 400 the arrow shows cartilage destruction
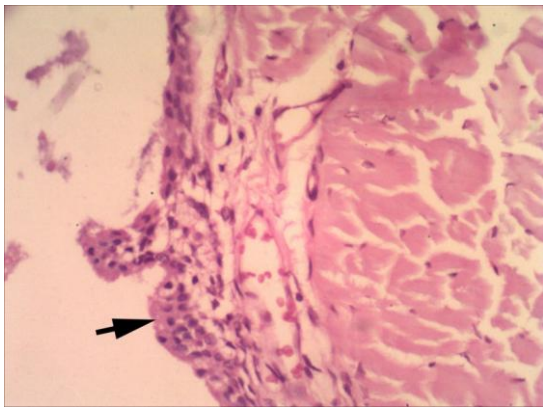

Figure 4: Right hind paw joint of atorvastatin group. X 400 arrow shows the fibrosis and inflammatory infiltrate
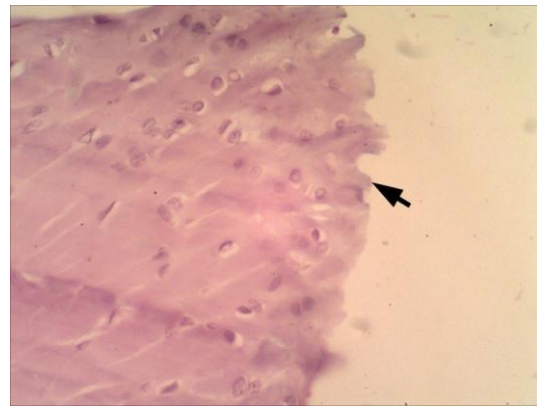

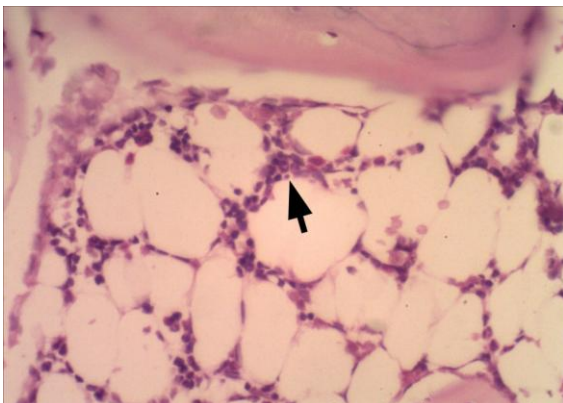**Figure 5:** Right hind paw joint of methotrexate group. X 400 showing cartilage destruction

Figure 6: Right hind paw joint of atorvastatin plus MTX group (full dose). X 400 the arrow points to the inflammatory infiltrate.

Figure 7: Right hind paw joint of atorvastatin plus MTX group (half dose) X 400 the arrow points to fibrosis.
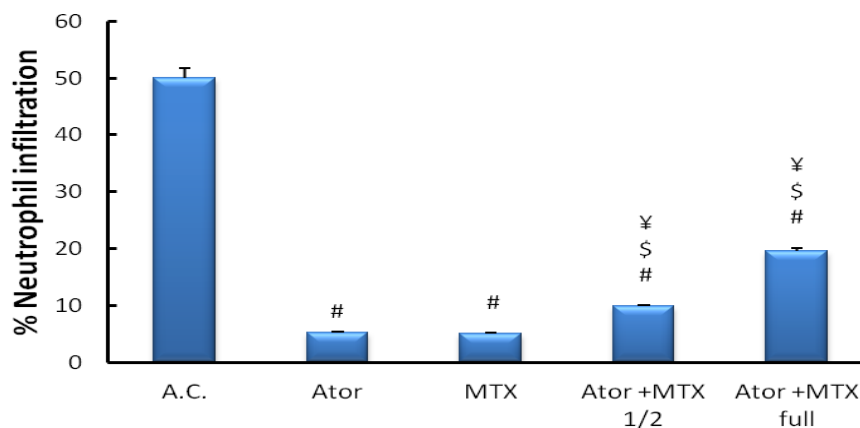
Fig. 8: Percent of neutrophil infiltration in the experimental groups. Monotherapy with atorvastatin or methotrexate or their combination significantly ameliorated the percent of neutrophil infiltration in arthritic rats. AC: arthritic control, Ator: atorvastatin, MTX: methotrexate. Results were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's test. [#] *P*< 0.05 *vs.* AC group, [$] *P*< 0.05 *vs.* atorvastatin group, [¥] *P*< 0.05 *vs.* MTX group. *n*= 6.
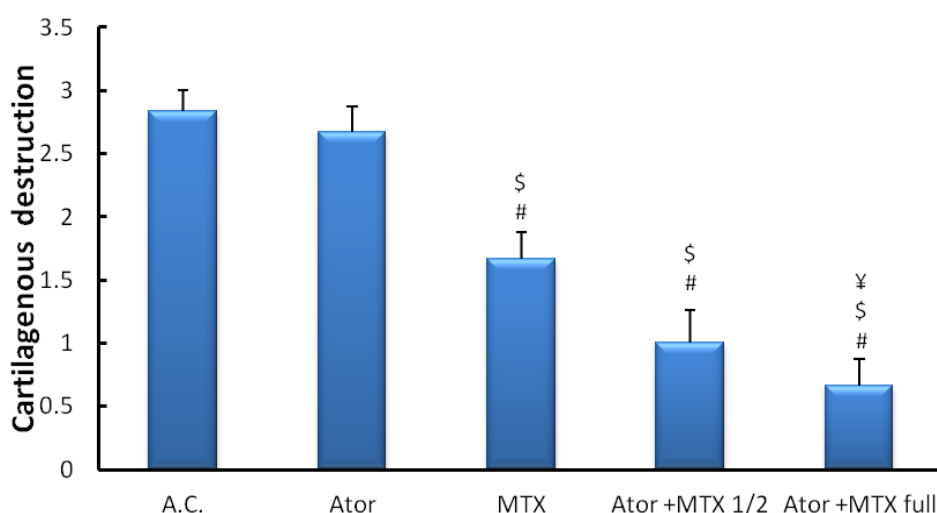

Figue 9: Score of cartilage destruction in the experimental groups. Monotherapy with methotrexate or its combination with atorvastatin significantly ameliorated the score of cartilage destruction in arthritic rats; also combination therapy in full dose significantly decreased this score as compared to monotherapy with methotrexate. AC: arthritic control, Ator: atorvastatin, MTX: methotrexate. Results were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's test. [#] *P*< 0.05 *vs.* AC group, [$] *p*< 0.05 *vs.* atorvastatin group, [¥] *P*< 0.05 *vs.* MTX group. *n*=6.

Since RA patients are more susceptible to cardiovascular diseases which is the most common cause of mortality in RA patients[15]; an increasing interest has aroused to study the effects of statins in improving RA. Adjuvant-induced arthritis in rats is a well established experimental model that has features similar to the human RA. It is characterized by chronic proliferative and inflammatory reactions in synovial membranes, producing pain, disability and eventually destruction of joints[16]. TNF-α, a potent cytokine with diverse effects on multiple cell types, is produced by monocytes,

macrophages, lymphocytes, and other cells. TNF-α is known to stimulate migration of lymphocytes to inflammatory sites such as the inflamed joint[17]. The anti-inflammatory effects of MTX in the current study were supported by many previous studies. Hildner et al. showed that MTX suppresses the production of both TNF and IFN-γ by T-cell-receptor-primed T lymphocytes from both healthy human donors and RA patients[18]. Braun and Rau stated that MTX seems to even prolong the life span of patients who tolerate the drug and have clinical benefit from this therapy[19]. The use of MTX has been limited by some of its toxic manifestations, such as abdominal discomfort, alopecia, oral ulcerations, and cytopenia[20]. Therefore, studies were made to use it in combination regimen- like the present study -to reduce the dose and for better effect. This agrees with many previous experiments. Bauerova et al. investigated the use of substances with anti-oxidant effects (coenzyme Q10 (CoQ10), carnosine, stobadinedipalmitate and pinosylvin) combined to MTX. All combinations tested showed a higher efficacy in affecting biochemical or immunological parameters than MTX administered in monotherapy[21]. The anti-inflammatory effects of statins in the current study was supported in several previous studies[22,23]. Also, statins modify apoptosis in smooth muscle, endothelial cells and synovial cells[24] and bones[25], for instance, statins may have preserving effects on periarticular bone in RA joints. Their beneficial role in murine collagen-induced arthritis[26], and refractory rheumatic disease was also proved[27].The first placebo controlled study was conducted by McCarry et al. to investigate therapeutic effects and vascular risk factor modification of statins in RA patients. It showed a significant reduction in the disease activity score in atorvastatin group compared to placebo group in patients with RA[28]. Our current study was in agreement with many previous human studies that showed that patients with RA taking statins; had lower CRP and lower swollen joint counts compared to patients not taking statins[29]. Our findings were not well-matched with other studies that detected inflammatory marker in RA patient before and after 20 mg atorvastatin therapy, while Lipid profile and arterial stiffness improved significantly[30]. Similar observation was made by Charles-Schoeman et al. with 80 mg atorvastatin in double blind placebo control trial. The changes in measures of clinical activity were not significant but there was a trend for a decrease in CRP during2 weeks of treatment with statin[31]. RA is still associated with an increased mortality mainly due to an increase in cardiovascular risk. Use of NSAIDs and glucocorticoids might be associated with an increased risk. The European League Against Rheumatism (EULAR) recommendations for cardio-vascular risk management in patients with rheumatoid arthritis and other forms of inflammatory arthritis are based on national guidelines regarding control of traditional cardiovascular risk factors[32]. Hence, the benefit of use of statins and its combination with MTX will allow for reduction of its dose and reduced side effects.

Conclusion

The study concludes that Statins are very benifecal for patients of RA especially if combined with DMARD.

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