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Research on the Effect of Tongxinluo on Macrophage Foam Cell Formation

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Abstract

Objective: To investigate the effect of Tongxinluo (TXL) on the foam cell formation of macrophages (RAW264.7). Methods: In vitro experiment: Cultured RAW264.7 mouse monocyte macrophages were divided into four groups: control group, model group, TXL treatment group, and DMSO group. A foam cell model was induced by incubating the macrophages with 50 μg/mL oxidized low-density lipoprotein (ox-LDL). The TXL treatment group was intervened with 100 μg/mL Tongxinluo. Lipid deposition within macrophages was observed using Oil Red O staining; cell proliferation activity was detected via a CCK-8 assay kit; and the levels of inflammatory factors (TNF-α, IL-1β, and IL-6) in the cell culture supernatant were measured by ELISA. Results: Compared with the control group, the model group exhibited a significant downregulation of macrophage proliferation activity, markedly increased lipid deposition, and a substantial increase in the release of inflammatory factors TNF-α, IL-1β, and IL-6 into the supernatant. In contrast to the model group, the TXL treatment group showed a significant upregulation of proliferation activity, markedly reduced lipid deposition, and a significant downregulation in the release of TNF-α, IL-1β, and IL-6. No significant changes in these indicators were observed in the solvent control (DMSO) group compared to the model group. Conclusion: Tongxinluo can significantly inhibit foam cell formation in macrophages and attenuate macrophage-mediated inflammatory injury.

Keywords

Tongxinluo; Macrophage foam cell formation; Inflammation; ox-LDL.

1. Research Background

As a leading cause of global mortality, stroke exhibits high incidence and disability rates that continue to show an upward trend annually. It represents one of the primary direct or indirect causes of brain death [1]. Ischemic stroke, the most prevalent type, is predominantly caused by injuries resulting from atherosclerosis (AS) [2]. Macrophages constitute one of the main cell types within atherosclerotic plaques [3]. Therefore, this study utilizes RAW264.7 macrophages to investigate the effect of Tongxinluo on macrophage foam cell formation in the context of atherosclerosis.

Atherosclerosis is a common, chronic inflammatory disease in daily life. Although its etiology remains unclear and cannot be fully elucidated by existing research, some scholars maintain that lipid deposition and inflammation are pivotal in its key pathogenic mechanisms [4].

During the formation of AS plaques, a substantial amount of endogenous low-density lipoprotein is progressively converted into oxidized low-density lipoprotein (ox-LDL), which accumulates extensively on the vascular endothelium, leading to the development of atherosclerotic plaques [5]. These plaques can reduce or obstruct blood flow. Monocytes that infiltrate the vascular wall differentiate into macrophages. Upon engulfing ox-LDL, these

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macrophages transform into foam cells [6] and concurrently express a plethora of inflammatory factors. The formation of foam cells marks the initiation of AS lesions [7].

Studies have indicated that Tongxinluo can reduce lipid deposition and the expression levels of inflammatory factors such as hs-CRP and IL-18 in a rabbit model of atherosclerosis [8]. Furthermore, experimental research has found that Tongxinluo capsules possess lipid-regulating, anticoagulant, anti-inflammatory, and antioxidant properties, protect vascular endothelium, and stabilize plaques, thereby potentially preventing and delaying the onset and progression of atherosclerosis [9]. Consequently, this study aims to investigate whether Tongxinluo can inhibit foam cell formation and the expression of inflammatory factors in mouse macrophages, seeking to provide therapeutic targets for atherosclerosis. It holds significant potential as a key factor in controlling the progression of atherosclerotic disease.

2. Materials and Methods

2.1. Experimental Materials

2.1.1. Cell Line

The murine RAW264.7 macrophage cell line used in this experiment was obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences.

2.1.2. Experimental Drug and Solution

Tongxinluo capsules were provided by Shijiazhuang Yiling Pharmaceutical Co., Ltd.

2.1.3. Experimental Reagents

The reagents utilized included an Oil Red O staining kit (Solarbio Science & Technology Co., Ltd., Beijing; Lot No.: G1346), a CCK-8 assay kit (Beyotime Biotechnology, Shanghai; Lot No.: C0037), and ELISA kits for TNF-α, IL-1β, and IL-6 (Calvin Biotechnology Co., Ltd., Suzhou; Kit No.: ck-120533).

2.2. Experimental Methods

2.2.1. Induction of Macrophage Foam Cell Model and Grouping

RAW264.7 cells were seeded into cell culture dishes. After 24 hours of culture in complete medium, the medium was replaced with serum-free medium for a 6-hour serum starvation period. The macrophage foam cell model was subsequently induced by stimulating the cells with 50 μg/mL ox-LDL for 24 hours. Intervention was performed by administering 100 μg/mL Tongxinluo to the foam cells. The cells were divided into the following groups:

(1) Control group: RAW264.7 murine macrophages cultured in complete medium.

(2) Model group: Cells subjected to 6-hour serum starvation in serum-free medium, followed by stimulation with 50 μg/mL ox-LDL for 24 hours.

(3) Tongxinluo administration group: After 6-hour serum starvation in serum-free medium, cells were co-treated with 50 μg/mL ox-LDL and 100 μg/mL Tongxinluo for 24 hours.

(4) DMSO group: Following 6-hour serum starvation, cells were co-treated with 50 μg/mL ox-LDL and an equivalent volume of DMSO for 24 hours.

2.2.2. Oil Red O Staining

(1) Cells were seeded into 24-well plates at a density of 1×10^5 cells/mL, with 500 μL of cell suspension per well, and allowed to adhere overnight in a cell culture incubator.

(2) Cells in each group received the corresponding interventions as described above.

(3) After intervention, the supernatant medium was carefully aspirated and stored in EP tubes for future use. Cells were washed twice with PBS, then fixed with fixative solution from the Oil Red O kit for 30 minutes.

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(4) The fixative was removed, and wells were rinsed twice with distilled water, followed by a 20-second immersion in prepared 60% isopropanol.

(5) After removing the isopropanol, pre-prepared Oil Red O staining solution was added to each well for a 20-minute incubation.

(6) The staining solution was discarded, and cells were briefly differentiated in 60% isopropanol for 30 seconds, followed by five washes with distilled water.

(7) Cell nuclei were counterstained with Hematoxylin for 2 minutes. After removal of the Hematoxylin, cells were washed five times with water. Cells were then covered with Oil Red O buffer for 1 minute before the liquid was aspirated.

(8) Distilled water was added to the wells, and lipid deposition was observed under a microscope.

2.2.3. CCK-8 Assay

(1) Cells were seeded into 96-well plates at a density of 1×10^4 cells/well in 100 μL of medium and cultured overnight.

(2) Cells were intervened according to their respective groups, with three replicate wells per group and blank control wells containing medium only.

(3) After intervention, the medium was removed and replaced with 100 μL of complete medium containing 10% CCK-8 solution. Plates were incubated at 37°C for 3 hours. Absorbance was measured at 450 nm using a microplate reader. Cell proliferation rate (%) was calculated as: [(Experimental group - Blank control) / (Control group - Blank control)] × 100%.

2.2.4. ELISA Detection

(1) Following treatment, cell culture supernatants were collected and stored at -80°C.

(2) All reagents were brought to room temperature before use.

(3) Standards were diluted according to the kit manufacturer's instructions.

(4) Three types of wells were set up: blank wells, standard wells, and sample wells. A total of 100 μL of serially diluted standards was added to the standard wells. 100 μL of standard diluent was added to the blank wells, and 100 μL of each sample was added to the sample wells. The plate was incubated at 37°C protected from light for 1 hour.

(5) The liquid was discarded, and 100 μL of Detection Antibody A working solution was added to each well. The plate was incubated for another hour at 37°C protected from light.

(6) After aspiration, each well was washed three times with 350 μL of wash buffer, ensuring complete removal of residual liquid after each wash.

(7) Freshly prepared 100 μL of Detection Antibody B working solution was added to each well, followed by a 30-minute incubation at 37°C.

(8) The liquid was discarded, and the plate was washed five times.

(9) A total of 90 μL of TMB substrate solution was added to each well. The plate was covered and incubated at 37°C protected from light for color development.

(10) The reaction was stopped by adding 50 μL of stop solution to each well. The optical density (OD) was immediately measured at 450 nm using a microplate reader.

2.3. Statistical Analysis

Statistical analysis and graphing were performed using SPSS 25.0 and GraphPad Prism software. Measurement data are presented as mean ± standard deviation ($\bar{x} \pm s$). Comparisons among multiple groups were conducted using one-way analysis of variance (one-way ANOVA). A p-value of less than 0.05 was considered statistically significant.

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3. Results and Analysis

3.1. Oil Red O Staining Results of Macrophages

As shown in Figure 1, observation of the four groups revealed no distinct lipid-like deposits within the macrophages of the normal control group. In contrast, compared to the control group, numerous red lipid droplets were observed in the macrophages of the model group. This finding sufficiently demonstrates that intracellular lipid accumulation can be induced by ox-LDL, indicating the successful establishment of the foam cell model. Compared to the model group, macrophages in the TXL treatment group exhibited a marked reduction in red lipid droplets. No significant difference in red lipid droplets was observed between the DMSO group and the model group, indicating that the solvent control exerted no therapeutic effect.

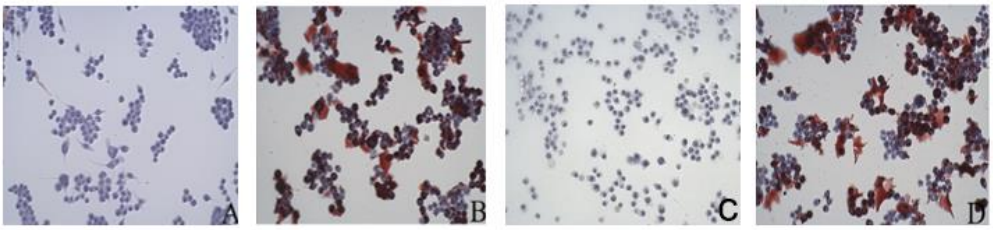

Figure 1. Oil Red O staining of macrophages (40× magnification)

Note: Panel A: Normal control group; Panel B: Model group; Panel C: TXL treatment group; Panel D: DMSO group.

3.2. CCK-8 Assay Results of Macrophage Proliferation Activity

As indicated in Table 1 and illustrated in Figure 2, the proliferation activity of cells in the model group was significantly decreased compared to the control group ($P < 0.05$). In contrast, the TXL administration group exhibited a marked increase in proliferation capacity relative to the model group ($P < 0.05$). No statistically significant difference in proliferation activity was observed between the DMSO group and the model group.

Table 1. One-way ANOVA of Cell Viability Across Experimental Groups ($\bar{x} \pm S$, n=3)

Group	Control **Group**	Model **Group**	TXL Treatment **Group**	DMSO Group
Cell Viability (%)	100 ± 2.23	28.40 ± 1.45*	69.12 ± 2.24#	28.19 ± 1.61

Note: Data are presented as mean ± standard deviation. *P < 0.05 compared with the Control Group; #P < 0.05 compared with the Model Group.

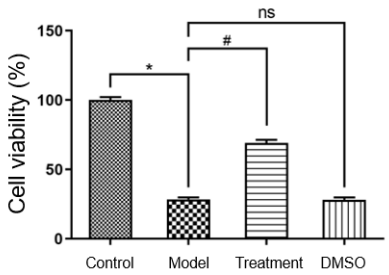

Figure 2. Viability of RAW264.7 Macrophages Across Experimental Groups

Note: *P < 0.05 compared with the Control Group; #P < 0.05 compared with the Model Group; ns: not statistically significant.

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3.3. ELISA Detection Results of Inflammatory Factor Release in Macrophages

As shown in Table 2 and Figure 3, the levels of inflammatory factors in the model group were significantly up-regulated compared to the control group ($P < 0.05$). In contrast, the TXL treatment group exhibited a significant reduction in inflammatory factor levels compared to the model group ($P < 0.05$). No significant differences in inflammatory factor levels were observed between the DMSO group and the model group.

Table 2. One-way ANOVA of Inflammatory Factor Levels by ELISA ($\bar{x} \pm S$, pg/ml)

One-way ANOVA of Inflammatory Factor Levels by ELISA ($\bar{x} \pm S$, pg/ml)

Inflammatory Factor	Control Group	Model Group	TXL Treatment Group	DMSO Group
IL-1β	170.28±6.22	307.12±8.76*	219.05±8.49 #	311.30±7.60
IL-6	283.22±11.17	545.45±8.00 *	328.25±8.01 #	549.81±15.91
TNF-α	1165.36±13.12	2608.44±48.05*	1472.53±42.69 #	2605.76±38.27

Note: Data are presented as mean ± standard deviation. *P < 0.05 compared with the Control Group; #P < 0.05 compared with the Model Group.

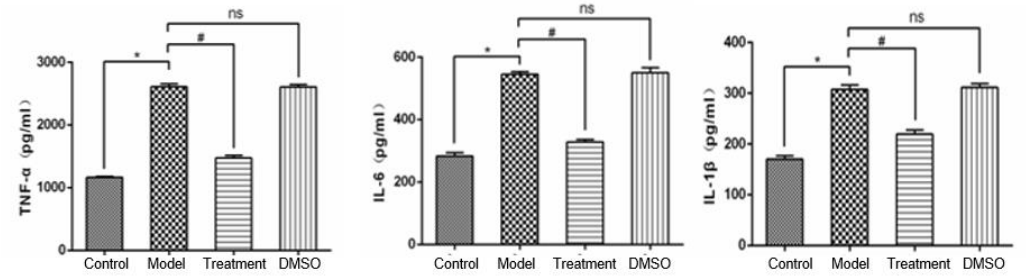

Figure 3. ELISA Results of TNF-α, IL-1β, and IL-6 Levels in Macrophage Culture Supernatants Across Experimental Groups

Note: *P < 0.05 compared with the Control Group; #P < 0.05 compared with the Model Group; ns: not statistically significant.

4. Discussion

The morbidity and mortality rates of ischemic stroke demonstrate an increasing trend, establishing it as a leading cause of disability among all diseases in China [10]. Atherosclerosis represents a fundamental pathological basis for ischemic stroke [11]. This study primarily investigates the effect of Tongxinluo (TXL) on macrophage foam cell formation and the inflammatory response, using this process as a key切入点. An *in vitro* foam cell model was established by treating macrophages with ox-LDL, followed by intervention with TXL. Oil Red O staining and CCK-8 assays confirmed that TXL intervention significantly suppressed ox-LDL-induced intracellular lipid deposition and markedly enhanced macrophage viability and proliferative capacity. ELISA results further demonstrated that TXL significantly inhibited the ox-LDL-induced inflammatory response in macrophages.

Research indicates that inflammatory responses are involved in the pathogenesis and progression of carotid atherosclerotic cerebrovascular disease [12-15]. Increased production of Interleukin-1β (IL-1β) and Interleukin-18 (IL-18), induced by the NLRP3 inflammasome, promotes the development of atherosclerotic plaques in animal models [16-18]. According to the Theory of Collaterals, atherosclerosis falls within the category of collateral stenosis or

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collateral stasis. Tongxinluo capsules, formulated under the guidance of this theory, aim to address the pathological changes of collateral stasis and contraction in cardio-cerebrovascular diseases. Its composition is based on the principles of supplementing Qi, promoting blood circulation, and expelling wind to unblock collaterals.

The formula comprises herbs for unblocking collaterals, including *Panax ginseng* (Ren Shen), *Hirudo* (Shui Zhi), *Scorpio* (Quan Xie), *Paeoniae Rubra Radix* (Chi Shao), *Cicadae Periostracum* (Chan Tui), *Eupolyphaga* (Tu Bie Chong), *Scolopendra* (Wu Gong), *Santalum Albi Lignum* (Tan Xiang), *Dalbergiae* Odoriferae *Lignum* (Jiang Xiang), *Olibanum* (Ru Xiang, processed), *Ziziphi Spinosae Semen* (Suan Zao Ren, stir-fried), and *Borneolum Syntheticum* (Bing Pian), characterized by their action of facilitating collateral circulation [19]. In the formula, *Panax ginseng* serves as the sovereign herb to tonify collateral Qi; sufficient Qi ensures vigorous blood movement, thereby naturally facilitating collateral patency. *Hirudo* and *Scorpio* act as minister herbs to resolve stasis and expel wind to unblock collaterals. *Eupolyphaga* assists *Hirudo* in removing stasis from the collaterals; *Cicadae Periostracum* assists *Scolopendra* and *Scorpio* to extinguish wind and relieve convulsions, thereby alleviating collateral contraction. *Paeoniae Rubra Radix* cools the blood and disperses blood stasis, while also moderating the warm nature of *Panax ginseng*; *Ziziphi Spinosae Semen* nourishes the blood and calms the spirit to prevent damage from stasis-removing herbs, serving as assistant herbs. *Santalum Albi Lignum*, *Dalbergiae Odoriferae Lignum*, *Olibanum*, and *Borneolum Syntheticum*, with their aromatic properties, guide the other components to penetrate the collaterals and orifices, acting as envoy herbs [20]. The combination of these herbs tonifies heart Qi to address the deficiency, while promoting blood circulation, unblocking collaterals, expelling wind, and relieving convulsions to eliminate pathogenic factors. Sufficient Qi and smooth blood flow ensure the patency of the heart and brain collaterals, thereby alleviating clinical symptoms.

This study, by establishing an *in vitro* macrophage foam cell model, demonstrated that 100 μg/mL TXL effectively inhibits foam cell formation and inflammatory injury in macrophages, a key cell type in atherosclerosis. Following TXL intervention, compared to the model group, the TXL treatment group exhibited reduced red-stained lipid droplets in the cytoplasm, increased cell proliferation viability, and downregulated levels of inflammatory factors. These findings sufficiently demonstrate that TXL inhibits lipid deposition and the release of inflammatory factors in foam cells.

Therefore, consistent with previous studies [21], TXL possesses anti-inflammatory and anti-lipid deposition properties, can promote the regression of atherosclerosis, and may serve as a potential targeted therapeutic agent for AS.

In summary, this experiment validates the efficacy of TXL in alleviating lipid deposition and inflammation, specifically demonstrating its significant inhibitory effect on macrophage foam cell formation in the context of atherosclerosis.

5. Conclusion

This study established an *in vitro* cell culture model to investigate the effects of Tongxinluo on a macrophage foam cell model. Utilizing Oil Red O staining, CCK-8 assay, and ELISA, we observed the therapeutic efficacy of Tongxinluo in reducing lipid deposition and mitigating the inflammatory response in macrophages.

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