**Correlation between the lymphocyte-monocyte ratio and cytokines in chronic inflammation in rats treated with allogeneic mesenchymal stem cells**

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Abbreviation:

MSCs - mesenchymal stem cells

Car - λ-carrageenan

WBC - white blood cells count

LMR - lymphocyte-monocyte ratio

α-TNF - tumor necrosis factor alpha

IL 6 - interleukin 6

CRP – C-reactive protein

Abstract:

The chronic inflammatory process is a pathological condition characterized by an ongoing active inflammatory response and tissue destruction. Many studies show that chronic inflammation can play a severe role in various age-related diseases, including diabetes, cardiovascular, and autoimmune diseases. One of the important but poorly studied factors affecting the regulation of chronic inflammation is the regulatory activity of MSCs. In this regard, the study of mesenchymal stem cells preventing chronic inflammation in the experiment is an important area of modern pathology.

On the one hand, increased cytokines, such as α-TNF, IL 6, and CRP, are reliable tools in diagnostic different inflammatory processes, especially chronic inflammation. On the other hand, nowadays, we need a more straightforward and not so expensive criterion for this purpose, for instance, a common total blood count and lymphocyte-monocyte ratio. For the first time, we investigated how trustworthy can be LMR and how possible to use it in chronic inflammation in rats to achieve prognostic goals.

This study investigated the correlation between α-TNF, IL-6, and CRP with LMR in rats' plasma in groups with chronic carrageenan inflammation and chronic inflammation with local injection of MSCs into the affected area. The study involved 132 adult male rats (180-220g), which were divided into groups. The inflammation model was chronic aseptic myositis caused by an intramuscular injection of 10mg λ-carrageenan (Sigma-Aldrich GmbH). Our experimental groups of rats were treated with MSCs (the injection into the inflamed site) in the amount of 1-2 million cells once. Blood sampling was performed from 6 hours to 28 days. We calculated our results using Statistica (data analysis software) version 13. For comparison, we used one-way ANOVA, Turkey's post hoc test, where p <0.05 was considered statistically significant.

 In our experiment, the correlation between levels of α-TNF, IL-6, and CRP with LMR in rats was described for the first time, demonstrating the suppression of chronic inflammation through MSCs.

Keywords: chronic inflammation, mesenchymal stem cells, lymphocyte-monocyte rate, tumor necrosis factor-alpha; interleukin 6; C-reactive protein.

Introduction:

The chronic inflammatory process is a pathological condition characterized by an ongoing active inflammatory response and tissue destruction. A significant number of immune cells, including macrophages, neutrophils, and eosinophils, are involved directly or through the production of inflammatory cytokines in the pathogenesis of chronic inflammation [1].

 It is well known from the literature that there is a general concept according to which chronic inflammation is the leading cause of cancer and aging processes [2]. Moreover, many studies show that chronic inflammation can play a severe role in various age-related diseases, including diabetes, cardiovascular, and autoimmune diseases [3]. One of the important but poorly studied factors affecting the regulation of chronic inflammation is the regulatory activity of MSCs.

 Human MSCs are individual progenitor cells that can be found in most vascularized body tissues. These cells have differentiation potential and are characterized by immunomodulatory and trophic activity [4].

 At first, this was met with great skepticism, then the immunomodulatory ability of mesenchymal stem cells was proven and well reproduced in experiments. [5, 6], and opened up the possibility of using mesenchymal stem cells for tissue replacement and regeneration and the treatment of immune-mediated and inflammatory diseases [7]. Thus, it was found that the implication of mesenchymal stem cells in the treatment of inflammatory diseases could give the most significant effect [8].

 It is the fact that there are many works devoted to the regenerative properties of mesenchymal stem cells [9–17], there are very few studies dedicated to the pathogenetic effect of mesenchymal stem cells on the processes of chronic inflammation [18, 19].

 In recent years, there has been a tendency towards an increase in type 2 diabetes, obesity [20], cancer [21, 22], which are a consequence of chronic inflammation and lead to early mortality and disability.

 In this regard, the study of mesenchymal stem cells preventing chronic inflammation in the experiment is an important area of ​​modern pathology.

It is well known that increased cytokines, such as α-TNF, IL 6, and CRP, are reliable tools in diagnostic different inflammatory processes, especially chronic inflammation [23]. (Fig. 1) Still, on the other hand, nowadays, we need a more straightforward and not so expensive criterion for this purpose, for instance, a common total blood count and LMR [24]. LMR is an important prognostic marker of endothelial dysfunction and inflammation. Low LMR correlates with worsening recovery and is probably a prognostic criterion for developing diseases associated with chronic inflammation.

In our study, we investigated how trustworthy can be LMR, and how possible to use it in chronic inflammation in rats to achieve prognostic goals.

 This study investigated the correlation between α-TNF, IL-6, and CRP with LMR in rats' plasma in groups with chronic carrageenan inflammation and chronic inflammation with local injection of MSCs into the affected area.



Fig. 1. Schematic of the relationship between TNF, monocytes, and macrophages. The positive feedback of TNF on monocytes for their functioning and then transforming into macrophages, which leads to enhancing the course of chronic inflammation

Materials and methods.

The study involved 132 adult male rats (180-220g), which were divided into groups. The inflammation model was chronic aseptic myositis caused by intramuscular injection of 10mg λ-carrageenan (Sigma-Aldrich GmbH) into the right hip [25].

The studies were carried out under the national "General Ethical Principles for Animal Research" (Ukraine, 2001) [26] (Strasbourg, 18.03.1986p.), the Declaration of Helsinki, (1964-2000), the charter of the Ukrainian Association for Bioethics and GLP (1992). Used the minimum acceptable for statistical processing and obtaining reliable results, the current number of animals (6 per group). Animals were sacrificed with inhalation of high concentrations of carbon dioxide (CO2), followed by decapitation.

Isolations of MSCs

We isolated MSCs from the rat femur's bone marrow using the standard method [27–30]. The bone marrow was withdrawn from the rat femur's epiphyses then washed with Hanks solution (Biowest, France). The cells were centrifuged to pellet (1000 rpm, 10 min). Mononuclear cells (Fig. 2) were obtained by centrifugation in a Ficoll-Hypaque gradient (density 1.077 g / ml) (Sigma, USA) at 400 g for 25 min and washed twice with Hanks solution (Biowest, France). After that, the cells were resuspended in physiological saline at a concentration of 1.0x106 in 1 ml [31, 32]. We measured the total number of cells with a cytometer by staining with 0.2% trypan blue solution (Janssen Chemica, Belgium). The structure of MSCs was investigated using a phase-contrast microscope; we studied cell cycles by flow cytometry. The immunocytochemical method studied MSCs phenotype.

Primary cultured MSCs were oval, fusiform, or polygonal, adhered to a plastic surface within 24 hours, and reached 90% confluence within eight days. After cleaning and breeding, they were equally long, spindle-shaped, and transmitted every five days. The adhesion rate was complete within 24 hours. Flow cytometry showed that 80% of fourth-generation MSC cells were in the G0 phase. Immunocytochemical analysis showed that MSCs were positive for CD29, CD105, CD166, VLA-4, and P-selectin, but negative for CD34 and CD45.



Fig. 2. MSCs of the femoral bone marrow of male rats.

Experiment and blood collection in rats

In the experiment, sixty rats had edema of the right thigh due to the intramuscular injection of λ-carrageenan. The other sixty rats were simultaneously injected not only with carrageenan but also with a suspension of MSCs. The control group had six intact rats without intervention and six rats that were injected with MSCs without inflammation.

The quantity of MSCs was 2 million cells in 0.4 ml per animal. There were ten terms in the experiment. For each assignment, we analyzed six rats with inflammation of carrageenan and six rats with inflammation plus MSCs. Animals were sacrificed under anesthesia after 6 hours on days 1, 2, 3, 5, 7, 10, 14, 21, 28. Blood samples were obtained by cardiac puncture. The blood smears were performed immediately (Fig. 3), and some blood was collected to the sterile tubes containing an anticoagulant (EDTA) for total blood count. Empty sterile tubes were used for plasma preparation. A blood clot appeared in 25-30 min, then the tubes were placed in a centrifuge and processed at 3000 rpm for 10 min. Plasma was obtained and sent to the freezer (-20ºC).



Fig. 3. Peripheral blood smears of rats.

Determination of α-TNF, IL-6, and CRP

Plasma α-TNF, IL-6, and CRP levels were measured using an enzyme-linked immunosorbent assay kit (Sigma-Aldrich GmbH) for quantitative measurement of target markers in biological fluids. We used ELISA for rat TNF-α, ELISA for rat IL-6, ELISA for rat CRP (C-reactive protein).

The lymphocytes monocytes ratio

LMR in rats was calculated in the same way as in humans. [33–40]. We took the absolute number of lymphocytes and divided them by the complete number of monocytes. As a result, we found a positive trend of LMR increasing. On day 21, LMR was significantly higher in the group of animals with chronic inflammation and MSCs.

Statistics

All calculations were performed using Statistica (data analysis software) version 13. For comparison, we used one-way ANOVA, Turkey's test, ere p <0.05 was considered statistically significant.

Results and discussion.

Elevated levels of proinflammatory cytokines accompany the majority of chronic inflammatory conditions. There are several therapeutic options for lowering these levels. These include monoclonal antibodies and cytokine receptor blockers, immunosuppressants, and non-steroidal anti-inflammatory drugs. None of these drugs are entirely safe or effective. Consequently, there is still a need to develop new approaches that can target other pathogenetic mechanisms. MSCs may be one such approach for decreasing the production of cytokines.

 The main objective of our study showed that the introduction of bone marrow MSCs in the area of chronic inflammation led to a significant decrease in proinflammatory cytokines, such as IL-6, TNFα, and CRP, in the plasma of animals of the inflammatory group plus MSCs. (Fig. 5,6,7). This decrease was statistically significant. LMR was significantly higher in animals with chronic inflammation and MSCs on the 1st day, then recovers on the 5th day. Since the 7th day, it was always superior during the next days with a peak on the 21st day. (Fig. 4)

The immunosuppressive activity of MSCs can explain these results. MSCs can support many types of immune cells, including B cells, T cells, dendritic cells (DC), natural killer cells (NK), neutrophils, and macrophages [41]. Interaction mechanisms are based on cell-cell contact, working in conjunction with the secretion of soluble immune factors to induce MSC-regulated immunosuppression [42]. These specific modulators include immunomodulatory factors, cytokines, growth factors, modulate inflammatory responses, and immune balance profiles. MSCs can also regulate the inflammatory process and repair damaged cells and tissues by attaching to the inflammation area [43]. The integration of MSCs with inflammatory processes enhances and suppress the immune response and depends on the general state of the immune system [44]. Surprisingly, MSCs modulate immunosuppression only when they are initially stimulated by inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin- (IL-) 1 [45]. MSCs respond to inflammatory cytokines and produce immunoregulatory secretors that mediate the process of inflammation [46,47].

It is crucial to admit that, even though we used allogeneic bone marrow MSCs, there was a significant decrease in the cytokines in the plasma of animals in the control group plus MSCs compared to the control group. So, this can be explained by the immunomodulatory ability of MSCs [48]. Such a significant decrease in proinflammatory cytokines may indicate the non-immunogenic properties of allogeneic MSCs. This fact may be necessary in cases where it is impossible to obtain autologous MSCs.

Even though the understanding of the mechanisms of immunomodulation based on MSCs remains incomplete, the growing volume of data prompts further studies of the properties of MSCs and their practical application. We believe that our research could help develop pathogenetic treatments for chronic inflammatory and autoimmune diseases that do not have side effects.

Fig.4. The lymphocyte-monocyte ratio. The red line is the level of LMR in groups with the inflammation treated with MSCs. Blueline - lymphocyte-monocyte rate in the usual course of inflammation.



Fig 5. The levels of α-TNF (natural course of inflammation and inflammation treated with MSCs).



Fig 6. The levels of IL-6 (natural course of inflammation and inflammation treated with MSCs).

 

Fig 7. The levels of CRP (natural course of inflammation and inflammation treated with MSCs).

Conclusions:

MSCs in the experiment showed themselves able to mitigate the severity of the inflammatory response. MSKs also showed good immunomodulatory properties. The LMR can be reliably used as an isolated immunological measurement in chronic inflammation or along with IL-6, α-TNF, highly sensitive C-reactive protein. It was reliable to use LMR in the course of chronic inflammation in rats to achieve prognostic goals.

 There is an excellent potential for further research into preventing chronic inflammation using MSCs, including clinical investigations. We see a great potential in the future study of stem cells as immunomodulatory and anti-inflammatory agents for increasing the quality and longevity of human life.

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Conflict of interest: none.

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**Кореляція між лимфоцитарно-моноцитарний співвідношенням і цитокінами при хронічному запаленні у щурів, які отримували алогенні мезенхімальні стовбурові клітини.**

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Анотація:

Хронічний запальний процес - це патологічний стан, що характеризується триваючої активної запальної реакцією і руйнуванням тканин. Багато досліджень показують, що хронічне запалення може відігравати серйозну роль в різних вікових захворюваннях, включаючи діабет, серцево-судинні та аутоімунні захворювання. Одним з важливих, але маловивчених чинників, що впливають на регуляцію хронічного запалення, є регуляторна активність мезенхімальних стовбурових клітин. У зв'язку з цим вивчення мезенхімальних стовбурових клітин, що запобігають хронізації запалення в експерименті, є важливим напрямком сучасної патології.

З одного боку, підвищені цитокіни, такі як α-TNF, IL 6 і CRP, є надійними інструментами в діагностиці різних запальних процесів, особливо хронічного запалення. З іншого боку, зараз для цього потрібен більш простий і не такий дорогий критерій, наприклад, загальний аналіз крові і лімфоцитарною-моноцитарного співвідношення. Ми вперше дослідили, наскільки надійним може бути лимфоцитарно-моноцитарне співвідношення і як його можна використовувати при хронічному запаленні у щурів для досягнення прогностичних цілей.

У цьому дослідженні вивчалася кореляція між α-TNF, IL-6 і CRP з лімфоцитарною-моноцитарним співвідношенням в плазмі щурів в групах з хронічним каррагіненовим запаленням і хронічним запаленням з локальної ін'єкцією мезенхімальних стовбурових клітин в уражену ділянку. У дослідження були включені 132 дорослих щурів-самців (180-220 г), які були розділені на групи. Модель запалення представляла собою хронічний асептичний міозит, викликаний внутрішньом'язової ін'єкцією 10 мг λ-каррагинана (Sigma-Aldrich GmbH). Наші експериментальні групи щурів отримували одноразово мезенхімальних стовбурові клітини (ін'єкція в запалену ділянку) в кількості 1-2 млн клітин. Забір крові проводився від 6 годин до 28 днів. Ми розрахували наші результати, використовуючи Statistica (програмне забезпечення для аналізу даних), версія 13. Для порівняння ми використовували односторонній ANOVA тест, де p <0,05 вважалося статистично значущим.

 У нашому експерименті була вперше описана кореляція між рівнями α-TNF, IL-6 і CRP з лімфоцитарною-моноцитарний співвідношенням у щурів, продемонструвавши пригнічення хронічного запалення за допомогою мезенхімальних стовбурових клітин.

**Ключові слова:** хронічне запалення, мезенхімальні стовбурові клітини, лимфоцитарно-моноцитарна співвідношення, фактор некрозу пухлини альфа; інтерлейкін-6; С-реактивний білок.

**Корреляция между лимфоцитарно-моноцитарным соотношением и цитокинами при хроническом воспалении у крыс, получавших аллогенные мезенхимальные стволовые клетки.**

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Анотация:

Хронический воспалительный процесс — это патологическое состояние, характеризующееся продолжающейся активной воспалительной реакцией и разрушением тканей. Многие исследования показывают, что хроническое воспаление может играть серьезную роль в различных возрастных заболеваниях, включая диабет, сердечно-сосудистые и аутоиммунные заболевания. Одним из важных, но малоизученных факторов, влияющих на регуляцию хронического воспаления, является регуляторная активность мезенхимальных стволовых клеток. В связи с этим изучение мезенхимальных стволовых клеток, предотвращающих хроническое воспаление в эксперименте, является важным направлением современной патологии.

С одной стороны, повышенные цитокины, такие как α-TNF, IL 6 и CRP, являются надежными инструментами в диагностике различных воспалительных процессов, особенно хронического воспаления. С другой стороны, сейчас для этого нужен более простой и не такой дорогой критерий, например, общий анализ крови и лимфоцитарно-моноцитарное соотношение. Мы впервые исследовали, насколько надежным может быть лимфоцитарно-моноцитарное соотношение и как его можно использовать при хроническом воспалении у крыс для достижения прогностических целей.

В этом исследовании изучалась корреляция между α-TNF, IL-6 и CRP с лимфоцитарно-моноцитарным соотношение в плазме крыс в группах с хроническим каррагиненовым воспалением и хроническим воспалением с локальной инъекцией мезенхимальных стволовых клеток в пораженную область. В исследовании были включены 132 взрослых крыс-самцов (180-220 г), которые были разделены на группы. Модель воспаления представляла собой хронический асептический миозит, вызванный внутримышечной инъекцией 10 мг λ-каррагинана (Sigma-Aldrich GmbH). Наши экспериментальные группы крыс получали однократно мезенхимальных стволовых клеток (инъекция в воспаленный участок) в количестве 1-2 млн клеток. Забор крови производился от 6 часов до 28 дней. Мы рассчитали наши результаты, используя Statistica (программное обеспечение для анализа данных) версия 13. Для сравнения мы использовали односторонний ANOVA тест, где p <0,05 считалось статистически значимым.

 В нашем эксперименте была впервые описана корреляция между уровнями α-TNF, IL-6 и CRP с лимфоцитарно-моноцитарным соотношением у крыс, продемонстрировав подавление хронического воспаления с помощью мезенхимальных стволовых клеток.

**Ключевые слова:** хроническое воспаление, мезенхимальные стволовые клетки, лимфоцитарно-моноцитарная соотношение, фактор некроза опухоли альфа; интерлейкин-6; С-реактивный белок.