Potential Anti-inflammatory Impact of SIRT1 Activator on RAW 264.7 Cells: In Vitro study

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Abstract:

Background: Inflammation serves as the bedrock for a diverse range of physiological and pathological processes. Para-inflammation is the adaptive response that occurs when tissue stress or dysfunction occurs. SIRT1 is a sirtuin family member. Numerous biological processes, such as cellular senescence, apoptosis, oxidative stress and inflammation are significantly influenced by SIRT1-mediated deacetylation.

Aim: The objective of this study is to assess the anti-inflammatory properties of Sirtuin1 activator (SIRT1 aptamer) by employing RAW 264.7 macrophage cell lines via the inhibitory effect of it on tumor necrosis factor-α, interleukin-1β and interleukin-6.

Methods: Variable concentrations of SIRT1 aptamer were applied to cells. The viability of the RAW 264.7 cell line was examined for the cytotoxic effects of SIRT1 aptamer using tetrazolium bromide (MTT) assay. After seeding RAW 264.7 cells. The cells starved and pretreated with aptamer at concentration 1.98 µM,4 µM for 1hr, after those cells were exposed to LPS (10 µg/ml), Harvesting of the RAW 264.7 cell culture supernatants. In accordance with the manufacturer's guidelines, the concentrations of TNF- α., IL-1β and IL-6 were ascertained utilizing enzyme-linked immunosorbent assay kits.

Results: The half–maximal inhibitory concentration for SIRT1 aptamer was 1.98 µM. At concentrations 1.98 µM,4 µM of its the level of tumor necrosis factor-α was decreased significantly by ($P<0.05$, <0.005, respectively) compared to the group treated with LPS alone, the SIRT1 aptamer at concentrations of 1.9 µM and 4 µM effectively suppresses LPS-induced cellular inflammation (level of Interleukin-6) in the RAW264.7 cell line. This effect is considerably different from the group treated with LPS alone ($P<0.05$ and <0.001, respectively). there was a significant decrease in LPS-induced cellular inflammation in RAW 264.7 cells when SIRT1 aptamer was added to the LPS stimulated cells, as compared to the (LPS-only) positive control $p<0.001$.

Conclusions: The use of SIRT1 aptamer exhibited superior anti-inflammatory properties by a reduction in the generation of pro-inflammatory mediators TNF-α, IL-6 and (IL)-1β from RAW264.7 cells that were activated with LPS. This can result in its utilization as a highly promising biotherapy with notable anti-inflammatory characteristics.

Keywords : Inflammation, SIRT1 aptamer, TNF-α, IL-6, (IL)-1β

عبرالتقييم المختبري RAW264.7التأثير المحتمل المضاد للالتهابات لمنشط السرتوين1على خلايا

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الخلاصة

الخلفية: يعمل الالتهاب كأساس لمجموعة متنوعة من العمليات الفسيولوجية والمرضية. شبه الألتهاب هو الاستجابة التكيفية التي تحدث عند حدوث إجهاد الأنسجة أو خلل وظيفي. تعتمد هذه الاستجابة في الغالب على الخلايا البلعمية الكبيرة التي تبقى في الأنسجة وتقع بين الحالة الطبيعية للجسم ورد الفعل الالتهابي النموذجي. من المحتمل أن يكون لشبه الألتهاب دور في تطور الاضطرابات الالتهابية المزمنة التي لوحظت بشكل شائع في الأمراض البشرية المعاصرة سرتوين 1 هو أحد أفراد عائلة سيرتوين. تتأثر العديد من العمليات البيولوجية، مثل الشيخوخة الخلوية، موت الخلايا المبرمج، الإجهاد التأكسدي والالتهاب بشكل كبير بإزالة الأسيتيل بوساطة سرتوين 1

الهدف: الهدف من هذه الدراسة هو تقييم الخصائص المضادة للالتهابات لمنشط سيرتوين 1 (سيرت 1 أبتامر) من خلال استخدام خط خلايا RAW264.7 عن طريق التأثير المثبط له على كل من IL-6, IL-1 β, TNF- α

طرائق العمل: تم تطبيق تراكيز مختلفة من سرتوين 1 أبتامر على الخلايا. تم فحص قابلية نمو الخلايا بعد 72 ساعة لمعرفة التأثيرات السامة لسرتوين 1 أبتامرعلى الخلايا باستخدام تيترازوليوم بروميد (ام تي تي) فحص.تم زراعة الخلايا لاحقا بأطباق الزراعة الخاصة بالخلايا لمدة 24 ساعة بعدها تم تجويع الخلايا ثم أضافة الأبتمار بتراكيز4مايكرومولاري1.98 لمدة ساعة بعد ذلك تم تحفيز الخلايا بمادة عديد السكاريد الشحمي بتركيز10مايكروغرام لكل مليلتر لمدة 6ساعات بعدها تم جمع الوسط الطاف لعمل فحص الإيلايزا.

النتائج: كان التركيز نصف الأقصى المثبط لسرتوين 1 أبتامر هو1.98 مايكرومولاري. في تراكيز 1.98مايكرومولاري و4مايكرومولاري من سرتوين ابتامر كان هناك نقصان ملحوظ بمستويات وسائط الالتهاب الثلاثة عامل نخر الورم الفا ,انترلوكين 6,انترلوكين 1بيتا مقارنة بالخلايا التي تم معاملتها بمادة عديد السكاريد الشحمي وحدها.

الاستنتاج: أظهر استخدام سرتوين 1 أبتامر خصائص فائقة مضادة للالتهابات من خلال تقليل توليد وساط الالتهابات. هذا يمكن أن يؤدي إلى استخدامه كعلاج حيوي واعد للغاية مع خصائص مضادة للالتهابات ملحوظة.

الكلمات المفتاحية: الالتهاب، سرتوين 1 ابتامر, عامل نخر الورم الفا،انترلوكين 6,انترلوكين 1بيتا

Introduction:

The inflammatory mechanism is a series of organized and dynamic chain of immune responses comprising cellular and vascular events with specific humorous secretions [(1)]. Cytokines are soluble secreted proteins that are released by immune cells. High cytokine levels may be detected in patients with immune responses to an inflammation and cancer, whereas in homeostatic concentrations are usually low or undetectable [(2)]. It has been shown that these polypeptides act as immunomodulators by autocrine, paracrine as well as endocrine mechanisms produced by a diverse array of cells including macrophages, B lymphocytes, and T lymphocytes [(3)]. Nevertheless, an overabundance of synthesized a clear correlation between cytokine storm and tissue damage, as well as a negative prognosis for serious illnesses [(4)]. The various types cytokines include: Chemokines,

interferons, Interleukins. However, it has been established that proteins are released by cells other than leukocytes. In addition, interleukins have the ability to transmit signals between non-leukocyte cells, TNF, Colony-stimulating factors (CSF) [5]. Tumor necrosis factor-alpha (TNF-α) is an inflammatory mediator that plays a role in multiple pathophysiological processes, such as enhanced vascular permeability, vascular dilatation, and neutrophil chemotaxis [6]. The utilization of IL-6 as a biomarker is justified due to its pivotal involvement in the initiation and sustenance of the inflammatory response. The clinical measurement of interleukin-6 (IL-6) has been identified as a robust prognostic indicator for death in individuals with pancreatic and cardiovascular diseases for instance. Hematopoietic cells, such as monocytes, macrophages like microglia or Kupffer cells, and dendritic cells are the main sources of IL-1β. These cells produce IL-1β when their pattern recognition receptors (PRR) are activated by pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMP). Moreover, the alpha cells of the pancreas release IL-1β, which can be examined in individuals with diabetes and obesity. Recent clinical investigations have suggested that the involvement of epithelium and endothelial IL-1β in cardiovascular disease is significant [7]. At present, medicinal agents with anti-inflammatory properties can be classified into two primary classifications: steroids and non-steroids. Nevertheless, these medications lack complete therapeutic efficacy and are linked to various adverse consequences. Hence, it is imperative to investigate and uncover anti-inflammatory medications that exhibit enhanced efficacy while minimizing adverse effects. The recognition of lipopolysaccharide (LPS) by Toll-like receptor 4 (TLR4) in RAW 264.7 cell line triggers a series of signaling events that ultimately lead to the activation of inflammation and the generation of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6[8]. The silent information regulator sirtuin 1 (SIRT1) protein, which is a member of the sirtuin family and functions as a NAD+-dependent deacetylase, is a post-translational regulator that is involved in the modulation of inflammation [9]. Emerging research has shown significant associations between SIRT1 and inflammation, with modifications in SIRT1 expression and functionality being associated with inflammatory disorders. SIRT1 has gained significant recognition for its notable antioxidant and anti-inflammatory characteristics. Furthermore, the activation of immune cells triggers many signaling pathways that are closely linked to the action of SIRT1. Based on the available information, SIRT1-targeted anti-inflammatory treatments are garnering growing interest due to their potential clinical utility in the treatment of inflammatory disorders. A macrophage cell line (RAW 264.7 cells) was subjected to LPS stimulation in order to establish an inflammatory model. This model was subsequently employed to assess the anti-inflammatory efficacy of SIRT1activator and investigate its potential mechanisms of action including the inhibitory effect of it on the levels of the proinflammatory cytokines, TNF-α, (IL)-1β, and IL-6.

Materials and methods

-Materials:

The RAW 264.7 cell line provided by the American Type Culture Collection (ATCC) was used as a model system in the current study. The culture medium used to maintain the cell line is the RPMI-1640 medium (Capricorn, Germany). The LPS was obtained from Solarbio LIFE SCIENCES. Dimethyl sulfoxide (DMSO) was purchased from Thomas Baker, India. The MTT [3-(4, 5-dimethylthiazol-2-yl)-
2.5diphenyltetrazolium bromide] was from Sigma, USA, and the Trypan blue stain was

from Thermo Scientific. Phosphate buffer saline (PBS) (Capricorn, Germany). Fetal bovine serum was purchased from Capricorn, GmbH. Penicillin /Streptomycin solution (100x), and Trypsin –EDTA (0.05%) in DPBS(1x) were purchased from Capricorn, Germany. SIRT1activator was purchased from Bioneer Integrated DNA Technologies (IDT)-Korea. Mouse TNF-α (Tumor Necrosis Factor-alpha) ELISA Kit, Mouse IL-6 (interleukin6) ELISA kit, Mouse IL-1β (interleukin 1 Beta) ELISA kit were all purchased from Elabscience, USA.

Methods

1. Cell line and culture

The RAW 264.7 cells were cultured in 75cm^3 tissue culture flasks, maintained in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin/ streptomycin and incubated at 37°C in a humid 5% CO_2 environment. Then cells were seeded in a 96-well plate at a density of 5×10^3 cell/ml and incubated for 24hr.

2. Preparation of SIRT1 aptamer stock solution

It is prepared by dissolving 100 pmol of SIRT1 aptamer stock solutions powder (Bioneer, Korea) in nuclease-free water, then stored at -20°C until use.

3. Treatment of RAW 264.7 cells with SIRT1 aptamer

When cells reached 80-90% confluence. Then serial dilutions of SIRT1 aptamer (0.075, 0.15, 0.35, 0.75, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 µM) were made and applied on cultured cells to seek the suitable concentration would be used in the following experiments.

4. Cell viability assay (MTT assay)

The MTT assay was employed to determine the impact of the SIRT1 aptamer on the viability of RAW 264.7 cells. Each well in the 96-well plate was seeded with 5×10^3 cells suspended in 100 µL of culture medium and incubated for 72hrs at 37°C in a humidified 5% CO_2 environment. A 3mg/mL of MTT solution was prepared using phosphate buffer saline (PBS). Then 20µL of the MTT solution was applied to each well and incubated for 4hrs at 37°C. The medium containing MTT was subsequently discarded, and 100µL of dimethyl sulfoxide (DMSO) was applied to all the wells. Meanwhile, the plates were incubated in a light-deprived environment for approximately 30min. The optical density was read at 540nm using a Promega® microplate reader and adjusted for a background absorbance at a wavelength of 600nm [10]. After 72hrs of treatment, cells were subjected to nonlinear regression analysis to determine the (IC50).

5.Cell sample preparation for ELISA tests:

After seeding RAW 264.7 cells into 24-well plates at a density of 1 x 10^5 cells/ml for 24 h. The cells starved and pretreated with aptamer at concentration 1.98 µM,3.9 µM for 1hr, after those cells were exposed to LPS (10 µg/ml) for 6 hours, Harvesting of the RAW 264.7 cell culture supernatants. To obtain the supernatant, the collected medium was centrifuged at 1000g at 2-8 °C for 20 minutes the samples divided up and stored at -20 °C(≤1month). In accordance with the manufacturer's guidelines, the concentrations of TNF- α., IL-6 and IL-1β were ascertained utilizing cytokine enzyme-linked immunosorbent assay kits (ELISA kits; Elabscience, USA).

Statistical analysis:

All statistical analysis of SIRT1 aptamer were performed using the GraphPad prism software 9.2. IC50 was done by using the nonlinear curve fitting software prism pad software. Tumor necrosis factor (TNF-α), interleukin IL-6, interleukin IL-1β, data comparison across all groups within the same plate was

conducted using one-way ANOVA with Tukey test in GraphPad prism 9.2.

-Results

1. The determination of the half-maximal inhibitory concentration (IC50) value.

Different concentrations were used to treat the RAW 264.7 cells from (0.075-100 µM) of SIRT1 aptamer. After 72hr, IC_{50} of SIRT1 aptamer in RAW 264.7 cell line was 1.9µM as shown in figure 1 and table 1.

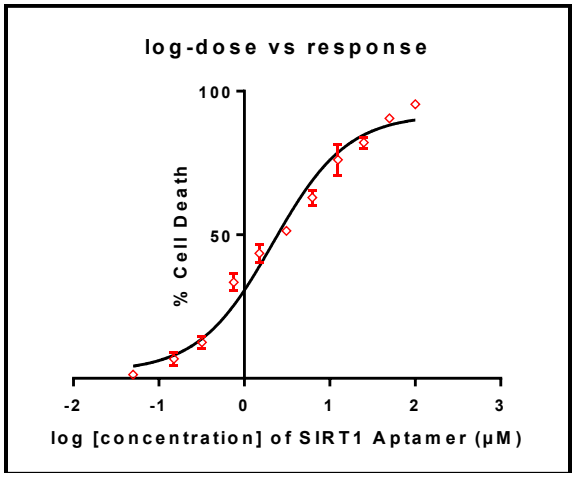

Figure 1: Dose-response curves for SIRT1 aptamer.

RAW 264.7 cells were treated for 72h with 0.075, 0.15, 0.35, 0.75, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 µM dose ranges of SIRT1 aptamer. The dose-response for SIRT1 aptamer was blotted over log transformed SIRT1 aptamer concentrations. The IC50 values were computed by nonlinear regression analysis using Prism Pad 8.1. The results indicate the standard error of the mean (SEM) for triplicate data.

Table 1: The average IC_{50} value, 95% confidence Interval and R^2 for RAW 264.7 cell line after 72h of SIRT1 aptamer treatment.

Incubation time	IC50 (µM)	HillSlope	CI at 95%(µM)	R^2
72h	1.9	0.643	1.626 to 2.866	0. 9788

2.Determination Inhibitory Impact of SIRT1 aptamer on Tumor Necrotic Factor Alpha (TNF-α) in LPS-Stimulated RAW 264.7 Macrophage Cell Line

RAW 264.7 cells were stimulated with 10 µg/mL LPS for 6 hours. Additionally, the cells were pretreated with different concentrations of SIRT1 aptamer: 1.9 µM ,4 µM. The SIRT1 aptamer at (1.9 µM ,4 µM) had a significantly greater inhibitory effect on the average level of TNF-α produced by LPS stimulated - RAW 264.7 cells compared to the group treated with LPS alone ($P<0.05$, <0.005, respectively), as seen in **Figure 2**

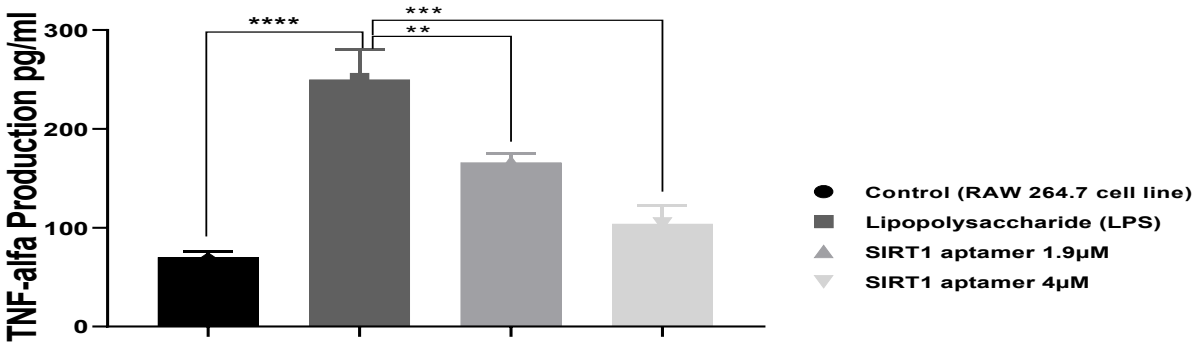

Figure 2: Inhibitory Impact of SIRT1 aptamer on Tumor Necrotic Factor Alpha (TNF-α).
TNF-α Productions in RAW 264.7 Cells Stimulated by LPS. Both concentrations of SIRT1 aptamer (1.9 and 4 μM) inhibited the cellular inflammations caused by LPS in RAW 264.7 cells. The values had been presented as the mean ±SD of three replications. (Prism Pad 9.2was used for the evaluation: one-way ANOVA with Tukey test). **** A comparison with control cells is made. Significant differences were observed when comparing the group treated with LPS alone to the groups with p**<0.05 and p***<0.001. According to Tukey test, means that are labelled with various stars indicate statistical significance at a significance level of $p < 0.05$.

3. Measuring Mouse Interleukin-6 (IL-6) levels in LPS-stimulated RAW264.7 Macrophages Cell Line.

The quantity of the proinflammatory marker Interleukin-6 (IL-6) was assessed to evaluate if SIRT1 aptamer can decrease proinflammatory responses generated by LPS in the RAW264.7 cell line. The cells were subjected to stimulation with 10 μg/mL of lipopolysaccharide (LPS) for a duration of 6 hours. In addition, the cells were pre-treated with varying doses of SIRT1 aptamer: 1.9 μM, 4 μM. **Figure 3** shows that the SIRT1 aptamer at concentrations of 1.9 μM and 4 μM effectively suppresses LPS-induced cellular inflammation in the RAW264.7 cell line. This effect is considerably different from the group treated with LPS alone (P<0.05 and <0.001, respectively).

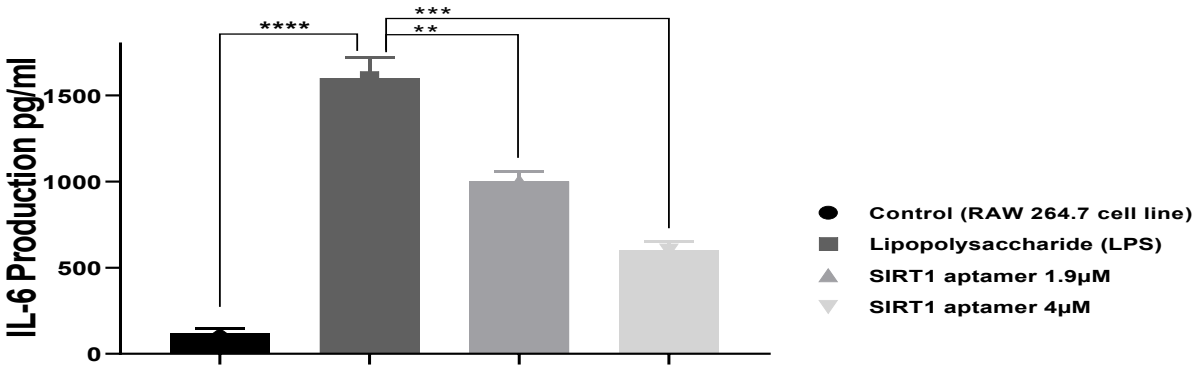

Figure 3: Inhibitory effect of SIRT1 aptamer on IL-6.
The generation of IL-6 in RAW 264.7 cells stimulated with LPS. The data are presented in the format of mean ± standard deviation (SD) of three replicates. ****Compare to control cells. When comparing the group treated with LPS alone, the difference was statistically significant with a p-value of p***<0.001, p**<0.05. By one-way ANOVA with Tukey test, means that are marked with distinct stars indicate statistical significance (p<0.05).

4. Quantification of Interleukin (IL)-1β on stimulated RAW 264.7 Macrophages Cell Line.

The cells were exposed to 10 µg/mL of lipopolysaccharide (LPS) for a period of 6 hours. Furthermore, the cells were subjected to different concentrations of SIRT1 aptamer prior to stimulation: 1.9 µM, 4 µM. As shown in **Figure 4**, there was a significant decrease in LPS-induced cellular inflammation in RAW 264.7 cells when SIRT1 aptamer was added to the LPS stimulated cells, as compared to the (LPS-only) positive control p <0.001, due to the anti-inflammatory properties of the aptamer against (IL)-1β.

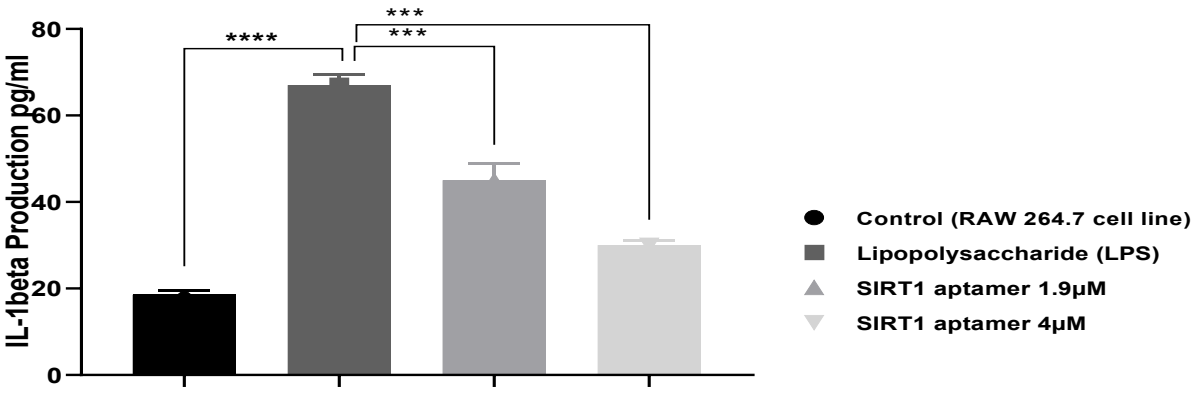

Figure 4: Inhibitory effect of SIRT1 aptamer on IL-1β.

Production of IL1β in RAW 264.7 cells stimulated with LPS. The format for values is mean±SD of three replicates ****Contrast with the control cells. The data was analyzed using one-way ANOVA and Tukey test with GraphPad prism 9.2. When comparing the group treated with LPS alone to the other group, the difference was statistically significant with a p-value of less than 0.005. According to Tukey test, means that are labeled with different stars indicate a statistically significant difference ($p<0.05$).

Discussion:

The process of phagocytosis in macrophages plays a crucial role in defending against foreign invasion and preserving homeostasis. Nevertheless, an overabundance of macrophages would significantly augment the process of phagocytosis and persistently secrete inflammatory cytokines, so exacerbating the harm inflicted upon the adjacent tissue [11,12]. Hence, the modulation of macrophages in maintaining homeostasis is a viable approach to mitigating aberrant immune responses characterized by chronic inflammation and tissue injury.

One of the studies conducted in this research aimed to investigate the anti-inflammatory properties of SIRT1 aptamer (SIRT1 activator**)** by *in vitro* cell culture. The SIRT1 dose-response curve shown in **Figure 1** demonstrates that SIRT1 aptamer decreased the viability of cells at the high concentrations (6.25, 12.5, 25, 50 and 100µM). This is suggested that the aptamer has no toxicity on cells at a concentration range below 6.25µM. Therefore, the 1.9µM was considered the half- maximal non-cytotoxic dose of SIRT1 aptamer and was selected for the subsequent experiments. Actually, by administering LPS to the RAW264.7 cells in our experimental model, we suggested an in vitro inflammatory model. The aforementioned condition resulted in a noticeable alteration in the cellular redox state, characterized by an excessive production of inducible proteins such as IL-1β, IL-6, and TNF-α. As numerous studies have demonstrated the significant anti-inflammatory properties of

SIRT1[13 -15]. LPS-stimulated cells treated with SIRT1 aptamer at concentrations 1.9 µM, 4 µM. The findings shown in **Figure 2, Figure 3** demonstrate the capacity of SIRT1 concentrations to elicit significantly greater inhibitory effect on the average levels of both TNF-α, IL-6 respectively produced by LPS stimulated - RAW 264.7 cells compared to the group treated with LPS alone. This finding is consistent with a previous study conducted by Suriyaprom *et al (2023)* The study revealed that mulberry leaf extracts and its components, resveratrol and oxyresveratrol (The function that is frequently mentioned and highly regarded is the potent activator of SIRT1) had significant anti-inflammatory effects by inhibiting the inflammatory reactions induced by lipopolysaccharide (LPS)[16,17]. Recent investigations have shown that the regulation of inflammation is interconnected through an antagonistic interaction between the NF-κB and SIRT1 signaling pathways [18]. In the same line, another investigation conducted by Hao Zhang *et al* (2017) Suppression of Sirt1 in mouse RAW264 cells macrophages boosted the expression of proinflammatory cytokines, including TNFα, IL-6, and IL-1β, in response to LPS stimulation. This increase was achieved via boosting the activity of NF-κB [19]. The targets of SIRT1, which are proteins that are not histones, are varied. Some examples include FOXO, PGC1-α, and NF-κB [20,21]. NF-κB is recognized as a primary controller of the inflammatory response because it has the ability to regulate the transcription of genes that play a role in the development of immunological and inflammatory responses. The canonical pathway primarily induced by cytokines such as TNF-α or IL1β, as well as toll-like receptor (TLR) agonists. An explicit connection between SIRT1 and the RelA/p65 subunit of NF-κB has been documented: SIRT1 can deacetylate lysine 310 of the RelA/p65 subunit, which impacts its ability to activate gene transcription. This leads to a decrease in the expression of genes that are involved in promoting inflammation [20,22]. For over assessing SIRT1 aptamer effects on pro-inflammatory cytokine another biomarker had been measured which was interleukin IL1β. In accordance with data available in Figure 4 the RAW264 cell lines, when exposed to LPS and treated with SIRT1 aptamer at concentrations 1.9 µM, 4 µM, exhibit a remarkable decrease in the expression of IL1β (p-value <0.005) comparing the group treated with LPS alone.

Conclusion:

According to the findings of this study SIRT1 aptamer capacity to restore the dynamic equilibrium of LPS-stimulated RAW 264.7 cell lines by a reduction in the generation of pro-inflammatory mediators TNF-α, IL-6 and (IL)-1β and so it put a stop to chronic pathological processes in the human body attributable to inflammation and its deleterious consequences make it an innovative candidate anti-inflammatory biotherapy and so, to safeguarding against chronic inflammation triggered by any cause.

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