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Research Article

Pharmacological Evaluation of Daidzein-loaded Solid Lipid Nanoparticle Gel for Anti-inflammatory Effect in Experimental Animals

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ABSTRACT

Daidzein (4′,7-dihydroxylisoflavone) is one of the major isoflavones present in various Leguminosae plants, including soybeans in higher percentages. Various researchers have reported daidzein for wide application in the management of inflammatory diseases. The objective of the present research work was to evaluate daidzein-loaded solid lipid nanoparticle (DAID–SLNs) gel for anti-inflammatory effectiveness in both acute and chronic inflammatory models, such as rat paw edema induced by carrageenan and granuloma induced by cotton pellets. Anti-inflammatory effects were observed by measurement of paw edema at different time intervals, level of proinflammatory markers, granuloma weight and histological study of granuloma tissue. Results of the study were showed that daidzein-loaded SLNs gel was gradually increased up to 180 minutes with an increase in percent inhibition of edema. DAID–SLNs gel was showed significant (p <0.05) effects in chronic inflammatory tests with reduced cotton pellet-induced granuloma weight in comparison to a control group and was comparable to market formulation Voltaren Emulgel. The anti-inflammatory effect was supported by significant (p < 0.05) attenuation of TNF-α (44.20 ± 1.55), IL-6 (35.28 ± 2.04), and PGE2 (48.24 ± 2.54) in comparison to the control group. In addition, the effect was supported by histological observation with many blood vessels that are growing and fewer acute inflammatory cells and lymphocytes. In conclusion, the anti-inflammatory effect of daidzein was observed by decreasing the exudate, granuloma weight, and inflammatory markers. The possible mechanisms could be the inhibitory effect on prostaglandin biosynthesis and expression of the inflammatory cytokines.

Introduction

The human body uses inflammation as a defense mechanism against damage and various types of infection. Inflammation is a complicated mechanism that involves several cell types and blood constituents. Abnormal inflammation can result in chronic discomfort, redness, swelling, stiffness, and damage to normal tissues. These inflammatory illnesses are caused by the immune system attacking the body's own cells or tissues.[1] When inflammation spirals out of control and destroys good tissue, it results in inflammatory diseases. Numerous inflammatory illnesses exist. Many, like joint inflammation in rheumatoid arthritis, happen when the immune system unintentionally causes inflammation when there isn't an infection. Others impact the entire body and arise as a reaction to trauma or tissue damage.[2]

About half of analgesics are anti-inflammatory medications, which treat pain by decreasing inflammation as opposed to opioids, which act on the central nervous system. By attaching to glucocorticoid receptors, many steroids, or more specifically, glucocorticoids, diminish swelling or inflammation. These drugs are generally considered under the category of corticosteroids.[3] NSAIDs (non-steroidal anti-inflammatory medications) reduce pain by inhibiting the enzyme cyclooxygenase (COX). The COX enzyme produces prostaglandins on its own, which leads to inflammation. All things considered, NSAIDs lessen or completely stop pain by preventing prostaglandins from

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ever being produced. Contrary to NSAIDs, which lessen pain and inflammation by blocking COX enzymes, a recent study found that paracetamol prevents endocannabinoids from being reabsorbed[4,5] it merely lessens discomfort, which probably explains why it has little impact on inflammation. NSAID use for an extended period of time can lead to stomach erosions, which can develop into ulcers and, in the worst situations, can cause serious bleeding that can be fatal. Additional risks associated with NSAIDs include aggravating asthma and damaging the kidneys. NSAIDs, both prescription and over-the-counter, increase the risk of myocardial infarction and stroke in addition to aspirin.[6] To overcome these major side effects, natural originated medicines is the choice for the treatment of inflammatory disorders.

Daidzein (4′,7-dihydroxylisoflavone) is an isoflavone subclass of flavonoid that is found widely in the Leguminosae plant and some traditional Chinese medicinal herbs such as Kudzu. Soybeans contain approximately 540 mg of daidzein per kilogram.[7] Daidzein has been linked to a number of biological activities, including an antiestrogenic effect (a weak estrogenic agent) through binding to the nuclear estrogen receptor, delaying the onset of diabetes, inhibiting of growth of cancerous cells, stimulation of osteoblast cell proliferation, and relief from menopausal symptoms. Daidzein is a strong contender for the treatment of cardiovascular disease and other inflammatory diseases, in addition to its positive effects on human health. Daidzein may lower low-density lipoprotein (LDL) cholesterol, increase nitric oxide generation, enhance vascular responsiveness, and suppress cell adhesion proteins, proinflammatory cytokines and platelet aggregation.[8]

However, daidzein's oral bioavailability is quite low, which restricts the extent of its therapeutic potential. Everyone agrees that the drug's physicochemical characteristics, such as its low solubility and robust metabolism in the liver and intestine, are responsible for its limited bioavailability.[9] In an effort to overcome this problem, a variety of microparticulate methods have been employed, including self-microemulsion and solid lipid nanoparticle drug delivery systems, which have shown that daizéin's bioavailability increased by 2.5 to 2.9 times fold.[10] The present research work aimed to prepare a daidzein-loaded solid lipid nanoparticle gel for effective anti-inflammatory action.

Material And Methods

Materials

Daidzein was procured from Yucca Enterprises, Mumbai. Carrageenan, glycerol monostearate and tween 80, carbopol 934, and polyethylene glycol 400 were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). The cytokine assay kit was purchased from TRANSASIA, Mumbai, India. All solvents, chemicals and reagents were used with analytical grade.

Preparation of Daidzein-loaded Solid-lipid Nanoparticles (DAID-SLNs) and DAID-SLNs Gel

Daidzein-loaded SLNs was prepared using the ultrasonication solvent emulsification method as described previously by Wang *et al*. (2022)[11] with slight modification. Based on the solubility study of daidzein, glycerol monostearate as the lipid phase and, methanol used as a solvent, Tween 80 as a surfactant were optimized with an objective to get an average size below 200 nm (data not shown). The desired amount of daidzein (50 mg) was dissolved in methanol at 70°C and added to a molten lipids mixture of glycerol monostearate and lecithin as the lipid phase. Lipids were melted at 10°C above their melting point. An aqueous solution of surfactant (2% Tween 80) was heated up to same temperature as the lipid phase and blended dropwise with the lipid phase under continued stirring. The resulting dispersion was sonicated using Ultra-Turrax® (IKA India Private Limited) for 10 minutes at 20000 rpm to get primary emulsion. This primary emulsion was subjected to a process for high-pressure homogenizer (1000 bars) up to 10 cycles to obtain finally daidzein-loaded SLNs by standing to allow the hot nanoemulsions at room temperature for cooling. The dispersion was filtered through a 0.45 µm filter in order to remove any impurities. A blank solid liquid nanoparticle was prepared by using a similar procedure without adding the daidzein. The detailed methodology of preparation and characterization of daidzein-loaded solid-lipid nanoparticles has already been submitted for publication in another journal that is part of previous research work.

Optimized DAID-SLNs dispersion was formulated into gel based on Carbopol 934 (1% w/w) as a gelling agent. After adding the calculated amount of freshly made DAID-SLNs dispersion and mixing for 10 minutes, drops of triethanolamine were added to adjust the pH to 6.5. The prepared gels were then left to remain for an additional night in order to release trapped air before being characterized.[12] Prepared DAID–SLNs loaded gel was characterized for pH, extrudability, viscosity, drug release and spreadability. Data of detailed methods and characterization is already submitted for publication in another journal. Further *in-vivo* investigation of the gel formulation for anti-inflammatory efficacy was conducted using acute and chronic inflammation models.

In-vivo Study for Anti-inflammatory Activity

Animals and protocol

Wistar albino rats (150–180 g) of either sex were selected for the *in-vivo* study for anti-inflammatory effect and all animals were placed in animal houses in separate polypropylene cages. Every animal had

unrestricted movement access to water and food. Before experimentation, all animals were kept on a 12:12 hours light: dark cycle and were acclimated to conventional laboratory settings of temperature (22 ± 3°C). The protocols used for the experiments and the care of the animals followed by CPCSEA/IAEC guidelines. Prior to dosing, the animals were kept in their cages for a minimum of 7 days to enable them to become acclimated to the laboratory environment. They are also randomly picked and marked to enable individual identification. The Institutional Animal Ethics Committee examined and approved the protocol of every experimental section (Reg. No. 1693/PO/Re/S/13/CPCSEA).

The animals were distributed into the following groups, each with six animals:

Group I was denoted as control group (without any treatment)

Group II was indicated as control group and applied gel base (without DAID–SLNs) 1 g daily topically

Group III, is treated topically with DAID–SLNs loaded gel (0.05% w/w), 1 g daily

Group IV, was indicated as standard and treated with Voltaren Emulgel (Novartis India Pvt Ltd) topically 1g daily

Carrageenan-induced paw edema

The rat paw edema produced by carrageenan was used to assess the acute anti-inflammatory effect using method of Patil *et al*, 2019)[13] with slight modification. Carrageenan solution (0.1 mL of 1%) was made in in physiological saline that was injected into the subplantar tissues of the left hind paw of each rat to produce edema.

The paw was dipped in mercury up to the mark, and a marker was used to mark it up to the ankle's hairline. A plethysmograph was used to measure the paw volume using the mercury displacement method at 30, 60, 120, 180, and 240 minutes. The carrageenan control group and the drug-treated group were compared for percentage inhibition of paw volume. The usual dose of indomethacin was 10 mg/kg p.o. and used as standard for comparison.[14] Animals were divided into four groups before starting experimentation on the first day of the experiment, which starved overnight with water *ad libitum*. The control group was given topically gel base (without DAID–SLNs) 1 g daily, while the other test group received the test drug and the regular drug. 0.1 mL of 1% carrageenan solution was injected subcutaneously into the sub-plantar side of the left hind paw challenging the rats one hour after dosing. The paw volume of each rat was measured at ½, 1, 2, 3 and 4 hours after the challenge. Compared with basal volume, the rise in paw volume was measured as a percentage. The discrepancy between the mean values of paw edema value of the treated animals and the control animal group was calculated for each time interval and statistically tested. Paw edema was measured accurately and calculated in the terms of percent inhibition using the following equation:

$$\%\text{edema inhibition} = [1- (Vt / Vc)] \times 100$$

Where, Vt and Vc are represent to edema volume of daidzein-loaded solid lipid nanoparticles gel treated and control groups, respectively.

Cotton pellet induce granuloma model

A common method for evaluating the proliferative and transudative aspects of chronic inflammation is the cotton pellet-induced granuloma. Basis of the model is a study of granuloma tissue that is formed after implantation of the foreign body that occurs when compressed cotton pellets are implanted subcutaneously in animals. After few days, connective tissue that has not been differentiated and histologically gained cells are visible next to the fluid infiltration.

The animals were distributed into four groups, given unrestricted access to water, and fasted for the whole night. For the whole trial, the animals were housed in aseptic settings. Rats were given anesthesia with anesthetic ether one hour after the initial dosage and 20 mg of sterile cotton pellets were placed subcutaneously, one in each axilla and groin. The incisions were packed with sutured by sterile catgut.[15] Six additional days were spent on the medication and vehicle regimen. On the 8th day, the animals were sacrificed by over anesthesia, and the cotton pellets were surgically removed. After removing superfluous tissue, the pellets were dried at 60°C until their weight stabilized. The weight of the cotton pellet after its initial weight was subtracted was found to be its net dry weight. The average weight of the pellets from the control group, test groups, and standard were noted. The change in granuloma weight in terms of percent inhibition relation to the vehicle control was calculated and statistically assessed.

The following formula was used to determine the cotton pellet's weight gain as a percentage of inhibition:

$$\%\text{Inhibition} = \{Wc - Wd/Wc\} \times 100$$

Where, Wd represent to pellet weight of DAID–SLNs loaded gel treated group; Wc represents to the pellet weight of the control group

Measurement of inflammatory markers in granuloma tissue

Low-grade inflammation is related to a number of chronic illnesses, including cancer, auto-immune diseases, type-2 diabetes, obesity, peripheral/coronary artery disease, and chronic obstructive pulmonary disease. Oxidative stress (OS), which can cause inflammation or act as a secondary cause, is closely associated with inflammation. Thus, reliable biomarkers of these signaling pathways may make it possible to identify inflammation, track its progression, and assess the effectiveness of treatment.[16] Inflammatory

Table 1: Effect of daidzein-loaded solid lipid nanoparticles gel on carrageenan induced paw edema in rats

Animal groups	*Observed rat paw edema as Mean ± S.D. (% inhibition of paw volume)*				
	30 minutes	*60 minutes*	*120 minutes*	*180 minutes*	*240 minutes*
Control (without treatment)	0.78 ± 0.07	0.81 ± 0.05	0.93 ± 0.02	0.98 ± 0.03	0.99 ± 0.08
Control (gel base only)	0.76 ± 0.05 (2.56)	0.78 ± 0.07 (3.7)	0.86 ± 0.06 (7.5)	0.92 ± 0.04 (6.12)	0.98 ± 0.05 (1.01)
DAID–SLNs loaded gel	0.52 ± 0.06 (33.33)*	0.49 ± 0.05 (39.50)*	0.45 ± 0.03 (51.61)*	0.43 ± 0.12 (56.12)*	0.45 (54.54)
Standard (Voltaren Emulgel)	0.55 ± 0.01 (29.48)	0.52 ± 0.04 (35.80)	0.50 ± 0.01 (46.23)*	0.44 ± 0.05 (55.10)	0.45 (54.54)

Each value is the mean ± S.D. (n = 6). *p < 0.05 compared with control group. DAID–SLNs: Daidzein-loaded solid lipid nanoparticles

Table 2: Effect of daidzein-loaded solid lipid nanoparticles gel on cotton pellet-induced granuloma in rats

Animal groups	*Granuloma weight (mg)*	*Percent inhibition*
Control (without treatment)	67.31 ± 2.74	-
Control (gel base only)	65.20 ± 2.61	03.13
DAID–SLNs loaded gel	31.85 ± 1.05	52.68
Standard (Voltaren Emulgel)	35.04 ± 1.13	47.94

Tabulated value represented as mean ± S.D. (n = 6). *p <0.05 when compared with a control group. DAID–SLNs: Daidzein-loaded solid lipid nanoparticles

markers such as prostaglandin E2 (PGE2), tumor necrosis factor-α (TNF-α), and interlukin-6 (IL-6) were examined in the granuloma tissue by ELISA Reader (Erba Lisa Scan EM, Mumbai). The assays were carried out in compliance with the manufacturer's suggested procedure.

Histopathological Study

After inducing granulomas in several groups of animals using cotton pellets, the granuloma tissue was removed and preserved in 10% formalin. After the usual preparation, very thick tissue sections (6 µm) were cut and stained with eosin and hematoxylin.[17] Different thin sections were selected for the qualitative assessment using a digital light microscope for the presence of inflammatory exudate, collagen fibers and area of granuloma and proliferated fibrous connective tissue were observed.

Statistical Analysis

All the results obtained from this study are expressed as mean ± SD. Each group contained six animals, which were considered to be statistically significant if p <0.05. The variation between mean values determined *via* one-way analysis of variance (ANOVA) and multiple comparisons using Tukey's test. Graphpad Instat Software was used for statistical analysis of the results.

Results And Discussion

Daidzein-loaded solid lipid nanoparticle gel was evaluated for anti-inflammatory potential using carrageenan-induced paw edema and Cotton pellet-induced granuloma models in rats. The results of both models were observed positive and detailed observations are discussed here.

Effect of Daidzein-loaded Solid Lipid Nanoparticles Gel on Carrageenan Induced Paw Edema in Rats

The acute anti-inflammatory activity was evaluated by carrageenan-induced edema in the hind paw of the rats. Carrageenan is a non-antigenic phlogistic agent that initiates the early phase of inflammation with devoid of any visible systemic effects. A total of 18 Wistar albino rats were used in the carrageenan-induced rat paw edema test. The effect of daidzein-loaded SLNs gel was gradually increased up to 180 minutes with an increase in percent inhibition of edema up to 56.12%. After 180 minutes time, the percent inhibition was decreased (54.54%) observed. This effect was observed significant in comparison to the control group of animals (Table 1).

The observations were suggested that the potential of the DAID-SLNs loaded gel to regulate or stop the production, release, or effect of histamine that involved in the inflammation may account for the efficacy of the treatment

Table 3: Effect of daidzein-loaded solid lipid nanoparticles gel on proinflammatory markers in cotton pellet-induced granuloma in rats

Animal groups	*TNF-α (pg/mL)*	*IL-6 (pg/mL)*	*PGE2 (pg/mL)*
Control (without treatment)	72.52 ± 2.84	54.29 ± 2.10	84.62 ± 3.61
Control (gel base only)	71.25 ± 2.64	52.39 ± 2.50	81.27 ± 3.24
DAID–SLNs loaded gel	44.20 ± 1.55*	35.28 ± 2.04*	48.24 ± 2.54*
Standard (Voltaren Emulgel)	48.28 ± 2.75*	41.52 ± 2.10*	51.62 ± 3.04*

Tabulated value is represented as mean ± S.D. (n = 6). * p < 0.05 when compared with control group. DAID–SLNs: Daidzein-loaded solid lipid nanoparticles

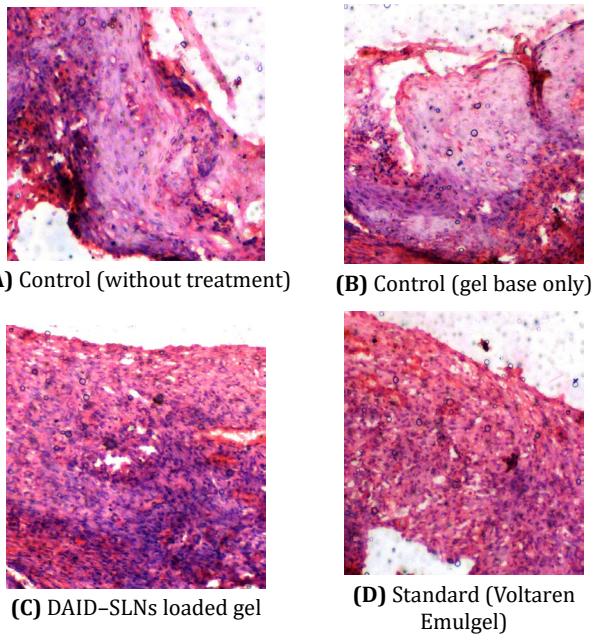

Fig. 1: Photomicrograph of granuloma tissue collected from different animal groups of cotton pellet granuloma model in rats: A) Control (without treatment); (B) Control (gel base only); (C) DAID–SLNs loaded gel; (D) Standard (Voltaren Emulgel)

for edema suppression. Chronic inflammation arises when the initial response is insufficient to completely eradicate the proinflammatory chemicals. Chronic inflammation is characterized by neutrophil infiltration and fibroblast proliferation together with fluid exudation. It happens as a result of proliferative cell formation, which can either spread or take the shape of granulomas.[18] The onset of edema occurs in two stages: in the first stage, which occurs 0 to 2.5 hours after the injection of carrageenan, inflammatory mediators such as histamine, serotonin, and bradykinins are released; in the second stage, which occurs 3 to 6 hours after the injection, prostaglandins are released.[19] In the present study, paw edema was greatly reduced by the DAID-SLNs loaded gel, suggesting that the daidzein and conventional gel may block the early phase of inflammation by inhibiting histamine and serotonin release. These observations confirmed that the prepared DAID–SLNs loaded gel may exhibit its anti-inflammatory effect by inhibiting the production, release or action of histamine.

Effect of Daidzein-loaded Solid Lipid Nanoparticles Gel on Cotton Pellet-induced Granuloma in Rats

A common tool for assessing the proliferative, transudative, and exudative aspects of chronic inflammation is the cotton pellet granuloma model. The weight of wet cotton pellet increases during the transudate period, and between 3 and 6 days, an inflammatory reaction to the cotton pellet implanted results in the creation of granulomas. Consequently, an increase in dry weight is thought to indicate the proliferative aspect of inflammation.[20,21] daidzein-loaded solid lipid nanoparticles gel showed potent anti-inflammatory properties in the chronic inflammatory model with reduced cotton pellet-induced granuloma weight significantly in comparison to the control group. The treatment with the market formulation Voltaren Emulgel also reduces granuloma weight significantly in comparison to a control group. The percent inhibition of granuloma by DAID–SLNs loaded gel was 52.68 (Table 2). The results thereby suggested the anti-inflammatory activity of daidzein-loaded solid lipid nanoparticles gel in the proliferative phase of the inflammation. The level of inflammatory markers, i.e., prostaglandin E2 (PGE2), tumor necrosis factor-α (TNF-α) and interlukin-6 (IL-6) were estimated in the granuloma tissue that represents to the inflammation status. The anti-inflammatory effect was supported by significant attenuation of TNF-α (44.20 ± 1.55), IL-6 (35.28 ± 2.04), and PGE2 (48.24 ± 2.54) in comparison to the control group (Table 3). One of the most important cytokine is TNF-α, which plays a major role in the pathophysiology of inflammation. Additionally, PGE2 is thought to be a significant modulator of several cytokines, which has been identified as an essential regulator of inflammation.[22] Based on the data, it is likely that the reduction of prostaglandin production is the mechanism of action of daidzein-loaded solid lipid nanoparticle gel for its anti-inflammatory effect.

Histopathological Observations

The most common method for getting access to the transudative, exudative and proliferative aspects of subacute inflammation is the cotton pellet granuloma approach. Results of the present study reveal that the tissue of control groups (without any treatment and gel base treated) have distinctive structural arrangement of the epidermis and dermis, fibro collagenous tissue with a high concentration of acute inflammatory cells, including neutrophils and lymphocytes (chronic inflammatory cells) and few growing blood vessels (Fig. 1). The area of granuloma infiltrated with prominent inflammatory exudate mixed with inflammatory cells, giant cells, macrophage, necrosis and exudates were also observed. DAID–SLNs loaded gel treated group, there were less acute inflammatory cells and lymphocytes and a greater number of blood vessels that were proliferating. The microscopical changes seen were comparable to those of the standard treated group, while the vascular modifications were fewer than those of the control.

Conclusion

The results of the study confirmed that the anti-inflammatory effect of daidzein was observed by decreasing the exudate, granuloma weight, and inflammatory markers. The possible mechanisms could be the inhibitory

effect on prostaglandin biosynthesis and expression of the inflammatory cytokines.

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