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Anti-inflammatory and analgesic activity of novel oral aspirin-loaded nanoemulsion and nano multiple emulsion formulations generated using ultrasound cavitation

Siah Ying Tang[a], Manickam Sivakumar[a,*], Angela Min-Hwei Ng[b], Parthasarathy Shridharan[a]

[a] Department of Chemical and Environmental Engineering, Faculty of Engineering, The University of Nottingham, Malaysia Campus, Jalan Broga, 43500 Semenyih, Malaysia
[b] Tissue Engineering Laboratory, Hospital Kebangsaan Malaysia, Kuala Lumpur, Malaysia

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ABSTRACT

The present study investigated the anti-inflammatory and analgesic activities of novel aspirin oil-in-water (O/W) nanoemulsion and water-in-oil-in-water (W/O/W) nano multiple emulsion formulations generated using ultrasound cavitation techniques. The anti-inflammatory activities of nanoemulsion and nano multiple emulsion were determined using the λ-carrageenan-induced paw edema model. The analgesic activities of both nanoformulations were determined using acetic acid-induced writhing response and hot plate assay. For comparison, the effect of pretreatment with blank nanoemulsion and reference aspirin suspension were also studied for their anti-inflammatory and antinociceptive activities. The results showed that oral administration of nanoemulsion and nano multiple emulsion containing aspirin (60 mg/kg) significantly reduced paw edema induced by λ-carrageenan injection. Both nanoformulations decreased the number of abdominal constriction in acetic acid-induced writhing model. Pretreatment with nanoformulations led to a significant increase in reaction time in hot plate assay. Nanoemulsion demonstrated an enhanced anti-inflammatory and analgesic effects compared to reference suspension while nano multiple emulsion exhibited a mild inhibitory effects in the three experimental animal model tests. The results obtained for nano multiple emulsion were relatively lower than reference. However, administration of blank nanoemulsion did not alter the nociceptive response significantly though it showed slight anti-inflammatory effect. These experimental studies suggest that nanoemulsion and nano multiple emulsion produced a pronounced anti-inflammatory and analgesic effects in rats and may be candidates as new nanocarriers for pharmacological NSAIDs in the treatment of inflammatory disorders and alleviating pains.

1. Introduction

Aspirin (acetylsalicylic acid) is a well-known non-steroidal anti-inflammatory drug (NSAID) widely used for its anti-inflammatory, anti-pyretic, analgesic, and platelet anti-aggregation properties (Awtry and Loscalzo, 2000; Amann and Peskar, 2002). It is always available over-the-counter in the form of tablets and capsules. Aspirin can commercially be synthesised via the esterification of the phenolic hydroxyl group of salicylic acid with acetic acid as a by-product. In plants, salicylic acid is synthesized from trans-cinnamic acid by carboxylation to benzoic acid (BA) and further 2-hydroxylation of BA to salicylic acid (Leon et al., 1995). After oral administration of aspirin, absorbed aspirin is rapidly de-acetylated to salicylate, and it was reported that the vast majority of circulating salicylate is bound to plasma proteins (Needs and Brooks, 1985). Similar to other NSAIDs, aspirin inhibits prostaglandin synthesis by inhibiting enzyme cyclo-oxygenases (also referred to as prostaglandin H synthase) early in the synthetic pathway. It works by acetylating the cyclo-oxygenases thereby irreversibly blocking the conversion of arachidonic to prostaglandin H_2, which in turn is metabolized by specific synthases or non-enzymatically to individual prostanoids (Vane, 1971; Vane and Botting, 1998). In fact, "inhibition of prostaglandin synthesis as the main mechanism of therapeutic action of aspirin-like drugs", demonstrated by Vane (1971), had further emboldened consumers on the established efficacy of aspirin, which in turn continues to make aspirin as one of the most popular over-the-counter medications as compared to paracetamol and ibuprofen. Although adequate inflammation and pain relief is achieved with the currently available aspirin dosage forms like tablets or capsules, some of their serious side effects in the gastrointestinal tract, kidney and platelets are major limitation to their routine use in therapy. For these reasons, there has been increased interest in search of safe analgesic and anti-inflammatory agents during recent years.

* Corresponding author. Tel.: +60 3 8924 8156; fax: +60 3 8924 8017.
E-mail address: Sivakumar.Manickam@nottingham.edu.my (M. Sivakumar).

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One of the promising approaches to minimize these adverse effects and enhance the efficacy of pharmaceutical active ingredient is to administer the drug via liquid nanoformulations, namely nanoemulsions and multiple emulsions. Nanoemulsions are known as isotropic dispersed systems with their droplet size in the size range of 50–500 nm (Solans et al., 2003; Capek, 2004). They are kinetically stable with long term stability with respect to creaming, sedimentation, and flocculation. Due to their small droplet size, nanoemulsions are expected to afford an efficient delivery of therapeutic agents to target sites in the body. However, to date, there have been limited investigations of nanoemulsions as an alternative drug delivery strategy to enhance the anti-inflammatory and analgesic properties of NSAIDs (Subramanian et al., 2008; Shakeel et al., 2009; Sakeena et al., 2010). Multiple emulsions are polydispersed yet complex dispersion systems in which the oil globules containing smaller water droplets are dispersed in an aqueous continuous phase. They are sometimes known as double emulsions, or "emulsions of emulsions". The development of such complex emulsions was carried out with the ultimate aim to facilitate the sustained release or transport of entrapped active aspirin drug, thereby reducing transient drug overload and gastrointestinal side effects caused by long-term administration of aspirin. In fact, multiple emulsion formulations have been widely studied in pharmaceutical field over the past decades where drugs including analgesic and antipyretic agents (i.e., antipyrine, 4-aminoantipyrine, diclofenac sodium, paracetamol, etc.) have been formulated into multiple emulsion for myriad of reasons including reducing local adverse effects (Khopade et al., 1998; Laugel et al., 1998), prolonged/controlled release (Khan, 2004; Lindenstruth and Müller, 2004), taste masking (Garti et al., 1983; Vaziri and Warburton, 1994) and drug targeting (Hino et al., 2000; Talegaonkar and Vyas, 2005). Oral administration of nanoformulations can possibly minimize the severity of drug-related side effects and reduce first-pass metabolism and thus maintains the plasma drug level for longer period of time. Hence, an improved aspirin nanoemulsion and nano multiple emulsion formulations with good stability could be useful in the treatment of inflammation and pain. Most importantly, both nanoformulations would be an efficient, convenient, flexible and more patient compliant approach in comparison to current drug tablets and capsules.

The aim of the present investigation is to examine the anti-inflammatory and analgesic effects of orally administered novel aspirin entrapped oil-in-water (O/W) and multiple water-in-oil-in-water (W/O/W) emulsion based nanoformulations and to compare their effects with the reference group (aspirin suspension). Both nanoformulations have been generated using ultrasound cavitation (horn transducer) under optimized conditions. It should be emphasized that acoustic cavitation; the formation, growth, and implosive collapse of bubbles is the main phenomenon responsible for droplet break-up and formation of fine droplets. The anti-inflammatory and analgesic studies were performed on rats by using carrageenan-induced rat hind paw edema model, acetic acid-induced writhing and hot plate assays to compare the nanoemulsions and nano multiple emulsions as well as suspensions containing aspirin. The present study is important for the evaluation of anti-inflammatory and analgesic properties of both the nanoemulsion and nano multiple emulsion generated using ultrasound cavitation technique since the use of both novel Cremophore EL based nanoformulations of aspirin have not been previously described in the literature.

2. Materials and methods

2.1. Experimental animals

Colony inbred strains of adult Sprague Dawley rats (male) of about 10–12 weeks of age (200–250 g), procured from Chenur Supply Sdn. Bhd. (Malaysia) were used for the pharmacological study. The animals were grouped and housed in large polyacrylic cages and maintained under standard laboratory conditions (temperature 22 ± 2 °C with dark/light cycle 12/12 h). The animals were allowed free access to standard pelleted diets (Glen Forest, WA, Australia) and clean fresh water in bottles ad libitum. The animals were acclimatized to the laboratory conditions for one week before the commencement of experiments. Each animal was used only once. All experimental protocols were in compliance with Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) on Research in Animals and the use of laboratory animals was conformed according to principles and guidelines to the ethical use of laboratory animals, UKMAEC, Laboratory Animal Resource Unit, Medical Centre, UKM.

2.2. Materials

Free samples of propylene glycol monolaurate Type II (Lauroglycol™90), diethylene glycol monoethyl ether (Transcutol HP®) were generously provided by Gattefosse Co. (Cedex, France). Aspirin (acetylsalicylic acid) was obtained from Fischer Scientific Co. Ltd. (Malaysia). λ-carrageenan and polyoxy 35 castor oil (Cremophor EL®) were purchased from Sigma–Aldrich Chemicals Company (Malaysia). Water used in all the preparation of formulations was obtained from a Milli-Q® Plus apparatus (Millipore, Billerica, USA). All other chemicals used were of analytical grade. Sodium chloride and acetic acid were purchased from Merck Sdn. Bhd. (Malaysia) and used in the biological assays.

2.3. Preparations of nanoemulsions and nano multiple emulsions

Unless otherwise stated, the optimized O/W nanoemulsion formulation was prepared using 200 mg drug, 10% w/w Lauroglycol 90, 10% w/w Transcutol, 3.52% w/w Cremophore EL, and 76.48% w/w de-ionized water, as previously described by author (Tang et al., 2011). The O/W emulsions was first pre-mixed for 15 min and then subjected to ultrasound for 70 s with 20% amplitude, which results into an emulsion with the mean droplet diameter and PDI (polydispersity index) of 215.6 nm and 0.289, respectively. For the preparation of W/O/W nano multiple emulsions, two-stage ultrasonic cavitational emulsification was employed. Based on the optimization results, the inner aqueous phase of optimal formulation comprised of 0.05% w/w Transcutol, 0.075% w/w Pluronic F68, 0.5% w/w glucose, 1% w/w gelatin and 0.5% w/w Cremophor EL. The primary emulsion was first prepared by adding the inner aqueous phase into an oil phase (a mixture of castor oil and Lauroglycol 90 at a weight ratio of 3:7), which consisted of 5% w/w Span 80 as lipophilic emulsifier. The resultant mixture was pre-emulsified using a vortex mixer for 5 min followed by first stage ultrasonication at 40% amplitude for 90 s using tapered microtip. In the second stage, freshly formed primary W/O emulsion was then re-emulsified with 0.5% Cremophore EL solution at 20% amplitude for 60 s to form the fine W/O/W multiple emulsion. The resulting mean droplet diameter was around 400 nm with PDI ranging from 0.3 to 0.4. It should be mentioned that the final concentrations of aspirin in the ultrasonically prepared O/W nanoemulsion and W/O/W nano multiple emulsion are 20 mg/ml and 10 mg/ml, respectively. For comparison purpose, blank nanoemulsion and reference aspirin suspension at the same drug concentration with aspirin-containing nanoemulsion were also prepared. Fig. 1A and B reveals that optimized aspirin-containing nanoemulsion and nano multiple emulsion droplets were almost spherical in shape with their homogenous nanometric size distribution. The STEM measured droplet size corroborates with the results obtained from the droplet size analysis using zetasizer. Both the optimal formulations of nanoemulsion and nano multiple emulsion that

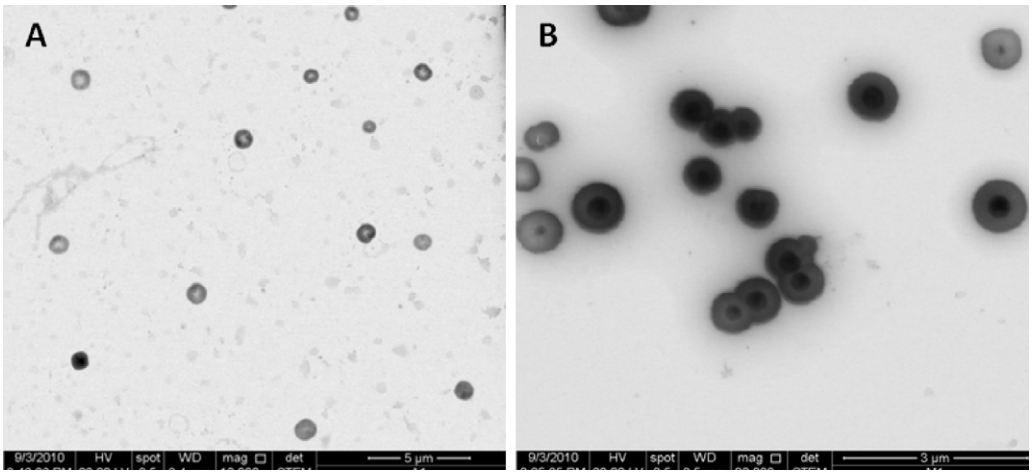

Fig. 1. Scanning transmission electron microscopy (STEM) photographs of optimized formulation of (A) aspirin-containing nanoemulsion and (B) nano multiple emulsion generated using ultrasound cavitation approaches.

contained aspirin have a good stability over one month of storage period.

2.4. Toxicity study

Toxicology test was performed according to the Organization for Economic Cooperation and Development (OECD) guidelines. This procedure helps in the usage of a minimal number of animals while allowing for acceptable data based scientific conclusion. Sprague Dawley rats selected by random sampling technique were divided into groups of three rats per group. The animals were fasted for 12 h with free access to water only. Nanoemulsion of aspirin was administered orally by means of bulb steel needle at a dose of 30 mg/kg initially and the animals were continuously observed for any changes in autonomic or behavioral responses for 3 days. The animals were kept under observation for 14 days to detect any mortality. If mortality was observed in two out of three animals, then the dose administered was considered as toxic dose. However, if the mortality was observed in only one out of three animals then the same dose was repeated again to confirm the toxic effect. If no mortality was observed, then only higher (60 and 90 mg/kg PO) doses of nanoemulsion of aspirin were employed for further toxicity studies. Experiments were repeated for nano multiple emulsion formulations with the same dose level of aspirin and toxicity sign was also observed for 14 days.

2.5. Inflammation study of λ-carrageenan-induced rat paw edema

The anti-inflammatory activity of nanoemulsion and nano multiple emulsion of aspirin was determined by the λ-carrageenan-induced edema test in the hind paw of rats according to a previously reported technique (Winter et al., 1962). Thirty Spray Dawley male rats were randomly divided into five groups (I–V) of six rats per group. They were fasted for 12 h and later treated as follows: group I were given normal saline (0.9% v/w, NaCI) (control), group II were given blank nanoemulsion (placebo) while groups III–V received suspension, nano multiple emulsion and nanoemulsion of aspirin at a dose of 60 mg/kg PO, respectively. A total of 0.1 ml 1% (v/v) suspension of λ-carrageenan in normal saline was injected intradermally into subplantar region of right hind paws of the rats. Each test compound was administered orally 1 h prior to the injection of λ-carrageenan. The paw width and thickness were measured before and at 1st, 2nd, 3rd, and 4th hour after injection of λ-carrageenan using digital vernier caliper. The paw volume was then calculated from width (a) and thickness (b) measurement using the following Eq. (1) (Giraldi et al., 1994):

$$\text{Volume} = \pi \times a^2 \times b \quad (1)$$

Increase in the paw volume of the right hind paws were taken as an indication of paw edema. The percentage inhibition of paw edema was calculated for each group with respect to its vehicle-treated control group by using the formula:

$$\text{Inhibition of paw edema} = \frac{V_c - V_t}{V_c} \quad (2)$$

where V_c represents an average increase in paw volume (average inflammation) of the control group of rats at a given time; and V_t is the average inflammation of the drug treated rats at the same time.

2.6. Acetic acid-induced writhing reflex

To evaluate the analgesic effects of the formulations, the method described by Sawadogo et al. (2006) was used with slight modifications (Sawadogo et al., 2006). The animals were divided into five groups of six animals per group. Group I served as a control and received normal saline (0.9% v/v, NaCI, p.o.), group II received blank nanoemulsion, and groups III–V served as treatment groups in which group III received aspirin suspension, groups IV and V received nanoemulsion and nano multiple emulsion of aspirin at the doses of 60 mg/kg, p.o. respectively. Acetic acid (1% v/v, 10 ml/kg bw) was injected intraperitoneally to all animal groups 60 min after the administration of prepared formulations. The number of writhes observed in each rat was counted for 10 min and recorded. A writhe is characterized by abdominal constriction and full extension of hind limb. A significant reduction of writhes in tested animals compared to those in the control group was considered as an antinociceptic response. The percentage inhibition of writhing was calculated using the equation (Dambisya and Lee, 1999):

$$\text{Inhibition of writhing} = \frac{N_c - N_t}{N_c} \quad (3)$$

where N_c is the average number of writhes of the control group, and N_t is the average number of writhes of the test group.

2.7. Hot plate test

The hot plate test was carried out according to the method described by Woolfe and McDonald (1944) which was adapted for rats (Woolfe and McDonald, 1944). Formulations to be tested were

administered. The hot plate was maintained at a fixed temperature of 55 ± 0.5 °C. The basal reaction time of all animals towards thermal heat was recorded. Animals were placed on the hot plate until it lifted one of its hind paws. Formulations to be tested were administered orally to five groups of animals 30 min before the thermal stimulus. The latency period (second) between placement and licking of hind paws or jumping was taken at intervals of 15 min for 60 min. A cut-off time of 30 s was fixed to avoid tissue damage to the paws. The data represent the mean reaction time for animals.

2.8. Statistical analysis

The values are represented as mean ± standard error of mean (S.E.M.). Statiscal difference between the control and treated groups were analyzed by one-way ANOVA followed by Tukey–Kramert's multiple comparison test. The differences were considered to be statistically significant at $p < 0.05$.

2.9. Histological analysis of λ-carrageenan-induced paw edema

After 24 h of λ-carrgeenan treatment, rats were sacrificed, and the tissue of the paws was removed and stored in 10% neutral buffered formalin prior to processing. The paw tissues were bisected longitudinally, placed in embedding cassets, embedded in paraffin wax, and then cut into 4 μm sections using a freezing microtome. The sections were stained with hematoxylin and eosin (H&E) for histopathological observation. A representative area was selected for qualitative light microscopic analysis of the inflammatory cellular response and images were collected with a 4× objective.

3. Results

3.1. Toxicity study

There was no significant change in autonomic or behavioral responses in rats treated with nanoemulsion and nano multiple emulsion formulations with the three different doses (30, 60 and 90 mg/kg, p.o.). No mortality was recorded in any group of animals up to 14 days.

3.2. λ-Carrageenan-induced paw edema

The results have been presented in Fig. 2. Nanoemulsion containing aspirin (60 mg/kg) presented the most remarkable inhibitory effect on carrageenan-induced paw edema by 37.6%. Nanoemulsion formulation showed a considerable reduction in the paw edema volume at 4 h after injection of inflammatory stimulus. Aspirin suspension has significantly inhibited the formation of edema similar to that of nano multiple emulsion formulation at the same oral administration dose. The former and latter presented an equipotent inhibitory effect by 16.6% and 17.3%, respectively 4 h after carrageenan injection. Both results were statistically significant (Fig. 1, $p < 0.05$). As expected, the blank nanoemulsion showed lowest inhibition of edema of only 10.6% after carrageenan injection.

3.3. Acetic acid-induced writhing reflex

As shown in Fig. 3, the mean number of writhing after i.p. injection of acetic acid was 4.3 ± 1.0 in control animals. A significant ($p < 0.05$) reduction in abdominal constriction was observed in nanoemulsion-treated rats and the mean value being 0.4 ± 0.2 (~91% inhibition). In contrast, blank nanoemulsion could produce 40% inhibition only in acetic acid induced writhing test. Also, at the same doses of aspirin, nano multiple emulsion and suspension

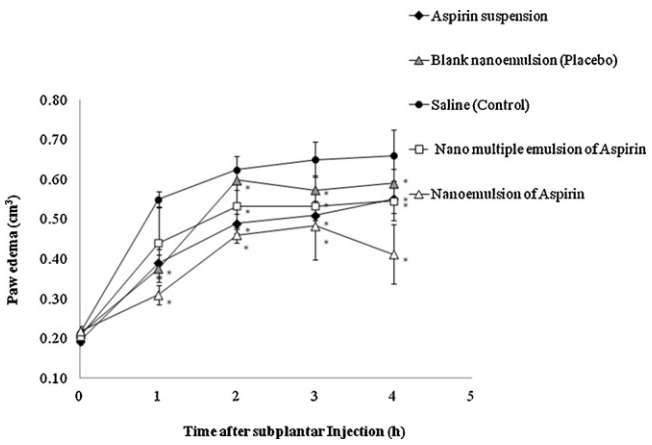

Fig. 2. Time-dependent inhibitory effect of the oral administration of nanoemulsion and nano multiple emulsion of aspirin against λ-carrageenan-induced paw edema in rats. Five groups of animals were pre-treated with the tested formulations 1 h before subplantar injection of 0.1 ml carrageenan (1%, w/v). Data are expressed as mean ± S.E.M. ($n = 6$/group). Asterisks denote significance levels when compared with control values (carrageenan). *$p < 0.05$.

caused a significant increase in inhibition of abdominal writhing, increasing it from 0% in control group to 63% and 81%, respectively ($p < 0.05$). This indicated that the reference drug suspension has slightly higher analgesic activity than the nano multiple emulsion used in this study.

3.4. Hot-plate test

The results presented in Table 1 show that the mean latency time was 4.46 s in vehicle (carrageenan) treated group at 60 min. At 60 min, nanoemulsion and nano multiple emulsion greatly increased the reaction time of animals towards the thermal sources compared with the control group. Surprisingly, the blank nanoemulsion also produced greater anti-nociceptive activity and the activity was comparable to that of reference drug suspension.

3.5. Histological observations on carrageenan-induced rat paw edema

Photomicrographs of sections stained with hematoxylin and eosin illustrate the carrageenan-induced inflammatory severity and the anti-inflammatory effect of nanoemulsion and nano multiple emulsion of aspirin on paw histology at 24 h after carrageenan injection (0.1 ml/paw). No inflammation, tissue destruction or

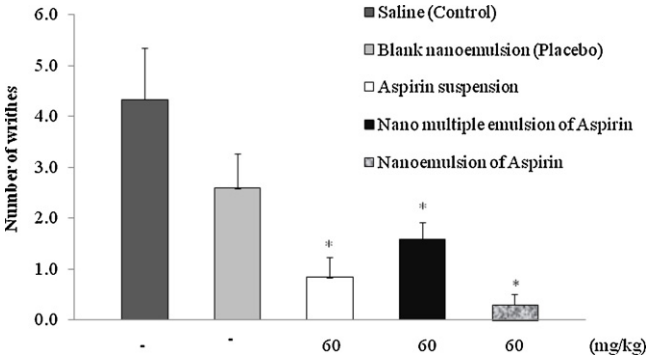

Fig. 3. The writhing test in SD rats consisted of the injection of 1.0% (v/v) acetic acid i.p. 30 min after oral administration of 0.9% (w/v) NaCl (control), blank nanoemulsion and tested vehicles. Data are expressed as mean ± S.E.M. ($n = 6$/group). Asterisks denote significance levels when compared with control values (λ-carrageenan). *$p < 0.05$.

Table 1
Effect of the oral administration of nanoemulsion and nano multiple emulsion of aspirin on hot plate test in rats.

Groups	Dose (mg/kg)	Latency period (s)				
		0 min	15 min	30 min	45 min	60 min
Saline (Control)	–	4.21 ± 0.37	4.84 ± 0.31	4.74 ± 1.44	5.05 ± 0.94	4.46 ± 1.38
Blank nanoemulsion	–	4.36 ± 0.43	6.01 ± 0.85	6.55 ± 1.81	9.15 ± 1.74*	8.11 ± 1.57*
Aspirin suspension	60	4.76 ± 0.43	5.03 ± 0.87	4.89 ± 0.34	8.25 ± 1.18*	7.86 ± 2.01*
Nano multiple emulsion	60	4.30 ± 0.33	4.92 ± 0.90	5.69 ± 1.07	6.89 ± 0.82	7.72 ± 1.16*
Nanoemulsion	60	4.85 ± 0.10	6.04 ± 0.91	9.01 ± 1.39*	9.37 ± 1.83*	9.37 ± 1.81*

Values expressed as mean ± S.E.M. ($n = 6$/group). Asterisks denote significance levels in comparison to control values.
* $p < 0.05$.

inflammatory cells influx were observed in the paws of normal rats (Fig. 4A, G, and L). In contrast, carrageenan-induced enlarged cavities resulting from tissues erosion were observed; these cavities were heavily populated by infiltrating cells (neutrophils) (Fig. 4B, H, and, M). Treatment of animals with nanoemulsion containing aspirin (60 mg/kg, 30 min prior, i.p.) clearly inhibited the leukocyte infiltration (Fig. 4F, J, and Q). However, administration of blank nanoemulsion had little effect on the inflammatory response (Fig. 4C, I, and N). As expected, the treatment with nano multiple emulsion produced a significant decrease in the number of cellular infiltrates (Fig. 4E, K, and P), as well as did the aspirin suspension (60 mg/kg, 30 min prior, i.p, respectively), a reference drug solution (Fig. 4D, J, and O).

4. Discussion

Of the anti-inflammatory drugs currently in use, the aspirin tablets and capsules are the most potent suppressor of inflammation. There are, however, many problems and sides effects associated with the long-term use of these dosage forms. In present study, aspirin was administered in the form of nanoemulsion and nano multiple emulsion as to enhance the drug efficacy and thus reducing the required dosage levels as well as to minimize the severity of drug-related gastrointestinal side effects. The present study shows that both the nanoemulsion and nano multiple emulsion containing aspirin had marked anti-edematogenic action in carrageenan-induced paw edema in rats. Acute inflammation process is characterized by an increase in vascular permeability, as a result of exudation of fluid and proteins, and conspicuously associated with polymorphonuclear cells migration (primary neutrophils) into the inflammatory site (Sherwood and Toliver-Kinsky, 2004). The carrageenan-induced rat paw inflammatory response is a widely used animal model test in the assessment of anti-inflammatory activity (Di Rosa, 1972; Garcia Leme et al., 1973). This technique has been extensively employed in the evaluation of anti-inflammatory action involving several chemical mediators such as histamine, serotonin, bradykinin, and prostaglandins (Vinegar et al., 1987; Garcia-Pastor et al., 1999). The temporal characteristics of the development of paw edema following subplantar injection of carrageenan has been characterized as biphasic event which involves various mediators in the generation of inflammatory response (Vinegar et al., 1969). The initial phase of edema (0–1 h) involves the release of histamine, 5-hydroxytryptamine (5-HT) and bradykinin and this phase is not inhibited by non-steroidal anti-inflammatory drugs (NSAIDs), as in this case aspirin (Di Rosa et al., 1971b). On contrary, at the second phase, accelerated paw swelling process takes place (1–6 h) due to elevated production of TNF-α, NO, and PGs (Posadas et al., 2004). The second phase has led to induction of COX-2, that generates iNOS and free radicals in the carrageenan-stimulated hind paw cells (Seibert et al., 1994) and the synthesis of COX-2 is dominantly triggered by pro-inflammatory cytokines.

Since the tested formulations did not produce any mortality in rats at a higher recommended dose of 90 mg/kg aspirin they may be considered to be relatively safe. The oral toxicity studies for nanoemulsions and nano multiple emulsions were also performed in mice and practically considered as non-toxic. Also, the results indicate that all the tested formulations presented a clear reduction in carrageenan-induced paw edema formation, where the nanoemulsion formulation exerts the highest anti-inflammatory

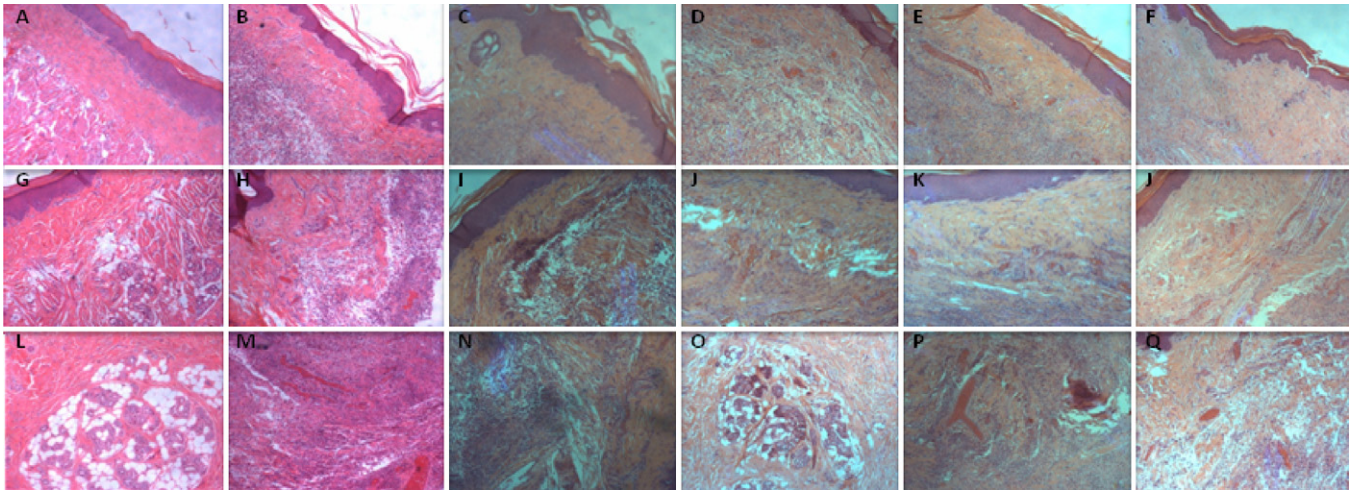

Fig. 4. Effect of nanoemulsion and nano multiple emulsion of aspirin administered on carrageenan-induced inflammation. Histological sections in the paw tissues at 24 h after the subplantar injection of carrageenan: normal tissue (A, G, L), carrageenan + saline (B, H, M), carrageenan + blank nanoemulsion (C, I, N), carrageenan + aspirin suspension (D, J, O), carrageenan + nano multiple emulsion (E, K, P), carrageenan + nanoemulsion (F, J, Q). Hematoxylin–eosin 20–40×, 4 μm sections. Six slices from each animal group were analyzed.

activity in the acute phase of inflammation. Further, the treatment with the nano multiple emulsion was also capable of suppressing the hind-paw edema, another important feature of this inflammatory study. In comparison to the aspirin suspension, as reference, a substantially higher effect was not observed in the nano multiple emulsion formulation, probably because of the slow inhibition for the effect of neutrophils migration. The blank nanoemulsion shows the lowest inhibitory effect against the λ-carrageenan-induced paw edema. Conversely, the nanoemulsion had the most powerful anti-acute inflammatory action, with the lowest paw edema volume presented at 4 h after the subplantar injection of carrageenan.

The inhibitory effect of the tested nanoformulations with the drug on neutrophil migration into the carrageenan-stimulated paw was confirmed by means of a histological analysis of the paw tissues. No histological alterations were observed in the paw tissues collected from normal rats. On contrary, marked inflammatory changes were observed including pronounced cellular infiltration in paw tissues collected from control group. The subplantar carrageenan-induced inflammation process resulted in a vast population of eroded tissue in rat hind paw. The oral administration of nanoemulsion, nano multiple emulsion and suspension containing aspirin caused significant pathological changes where there is less inflammatory cell infiltration in the rat paw tissues. There was a great reduction in the stained cells in slices of paw tissue, indicating fewer inflammatory cells in the paws of carrageenan plus nanoformulations-treated rats. This result clearly demonstrates that nanoemulsion and nano multiple emulsion containing aspirin have a marked anti-inflammatory potency, attenuating the influx of neutrophils which promote the cytotoxic tissue damage, following the injection of carrageenan. The anti-edema effects of nanoemulsion and nano multiple emulsion containing aspirin are primarily due to their inhibition of NO by inhibiting neutrophils. The anti-inflammatory mechanism of nanoemulsion may be linked to PGs and NO synthesis inhibition, similar to the anti-inflammatory mechanism of indomethacin in inhibiting the inflammatory activity induced by carrageenan (Di Rosa et al., 1971a). These findings show that the aspirin involved in the inhibition of the cellular migration are in lower recommended concentration, indicating a lower dose in the form of simple O/W nanoemulsion is able to produce a substantially higher effect in comparison to the other tested formulations.

In order to evaluate the antinociceptive property of any new substance using behavioral nociceptive tests it is essential to employ different tests which differ in stimulus quality, intensity and duration (Tjolsen et al., 1992). For this reason, nanoemulsion and nano multiple emulsion containing aspirin were tested for their antinociceptive effect employing two different nociceptive assay procedures. For chemical method, acetic-acid induced writhing assay (Koster et al., 1959) is used since it employs minimal noxious stimulus whereas for thermal method hot plate assay (Eddy and Leimbach, 1953) is adopted as it utilizes a high degree of thermal nociception. Formulations showing good antinociceptive effect in these two methods can be considered as potent analgesic agent. It should mentioned that acetic acid-induced abdominal constriction is an assay known to be mediated by peripheral receptors while hot plate assay is an indicative of central analgesic effect. The result of the present study shows a considerable reduction in the number of abdominal constrictions in acetic acid assay by pretreatment with nanoemulsion and nano multiple emulsion. This clearly indicates that the potent antinociceptive action of these two nanoformulations.

At a dose of 60 mg/kg, nanoemulsion exhibits the highest percentage of inhibition as compared to other tested formulations while nano multiple emulsion presented mild analgesic effect in this acetic acid-induced writhing test. With respect to the writhing test, Deraedt et al. (1980) reported that intraperitoneal administration of acetic acid not only induces the liberation of prostaglandin but also releases a high level of prostaglandins and sympathomimetic system mediators (Deraedt et al., 1980) like $PGE_{2\alpha}$ and $PGF_{2\alpha}$ during the first 30 min after injection. The antagonized mechanism for the abdominal constriction is postulated to be partially related to inhibition of prostaglandins and PGE_2 production in peritoneal fluid. Thus, the results obtained for the abdominal writhing assay using acetic acid are similar to those obtained for the edematogenic test using carrageenan. The antinociceptive effect of these two aspirin nanoformulations was also confirmed from the results of thermal nociceptive assay. A marked increase in reaction time was observed after treatment with the investigated drug nanocarriers indicating the efficacy of nanoemulsion and nano multiple emulsion in a model of thermal nociception. In this assay, nanoemulsion exerted the highest degree of inhibition of nociceptive among the other tested vehicles similar to a visceral pain model. This observation may suggest a more preferential and predominant effect of nanoemulsion formulation on analgesic activity. This rationale was confirmed when nanoemulsion was found to be the most active in the two nociceptive tests, indicating an improved analgesic activity. Overall, the inhibitory effects of both the nanoformulations containing aspirin in the two animal models suggest that both have peripheral and central analgesic activities. Furthermore, as no visible side effects or animal death were noted at a high dose of aspirin in both the writhing and hot plate assays and this observation was assumed to be the result of a specific pharmacological effect of nanoemulsion and nano multiple emulsion.

Although the experimental results showed that the therapeutic effect of aspirin suspension was comparable to nanoemulsions and even more effective than nano multiple emulsion, the difficulties encountered during the formulation, physical and chemical stability, taste masking and palatability greatly discount its function as a replacement for aspirin tablet and capsules. In this respect, nanoemulsion appears to be an ideal drug delivery system for the administration of aspirin as it is kinetically stable and often characterized by its increased drug solubility, rapid dissolution velocity, and enabling bioavailability after oral administration. The results for the group-treated with nano multiple emulsion did not differ significantly from those obtained for group-treated with reference suspension at the same oral dose tested.

This observation suggests a possible prolonged drug delivery due to the presence of rigid multilayered coating on the droplets that are responsible for the slow inhibitory action for the paw edema and thermal nociceptive effect, participating less on the anti-inflammatory and analgesic activity of the aspirin. This could be related to a possible specific compound interaction of viscoelastic gel-like membrane structure formed throughout the inner aqueous and the intervening oil phases which delay the release kinetics of aspirin. Since nano multiple emulsion is made up of different composition of several substances, the addition of these ingredients might be contributing to the observed anti-inflammatory and analgesic action of nano multiple emulsion, suggesting a probable synergism between several chemicals in the multi-compartment formulation. Thus multiple active components have also contributed to the slow anti-inflammatory activity of nano multiple emulsion. Besides, in both the nanoemulsions and nano multiple emulsion formulations, Transcutol, a well known drug penetration and permeation enhancer was used as an excipient. Thus, the different amount of Transcutol present in both the nanoformulations can also be used to explain the observed difference of therapeutic performance between the nanoemulsions and nano multiple emulsion. In the case of nano multiple emulsion, which has significantly lower concentration of Transcutol as compared to nanoemulsion, the pharmacological effect of aspirin following oral administration is expected to slower and hence the resulting anti-edematogenic and antinociceptive activity appeared

to be less pronounced. The slow inhibitory nature of nano multiple emulsion might also explain why the percentage inhibition of the reference drug suspensions were relatively higher than those complex systems in the above three assays of the present study. Furthermore, oral administration of aspirin in the form of nano multiple emulsion offered the advantage of being less invasive and less unpleasant odor compared to tablet and suspension since the drug is encapsulated in the inner core of nano multiple emulsion for masking unpleasant bitter taste and for prolonging the drug delivery.

This study demonstrated that nanoemulsion and nano multiple emulsion containing aspirin exhibited distinct anti-inflammatory activity against carrageenan-induced paw edema and analgesic activity against visceral pain responses triggered in rats by i.p. acetic acid injection and thermal nociception via hot plate assay. The anti-inflammatory mechanisms of these two nanoformulations are primarily accounted for the inhibition of neutrophil infiltration, iNOS and COX-2 protein expression. Furthermore, both had analgesic effect in both nociceptive models. The molecular pathways by which both formulations exert their analgesic effect were somehow similar or correlated to their anti-inflammatory effect, such as reducing the production of various pro-inflammatory cytokine and suppressing neutrophil activation. The anti-inflammatory activity produced by aspirin nanoformulations was the most remarkable and obtained at dose levels devoid of any side effects. In the carrageenan-induced inflammation test as well as in the two nociceptive assays, the anti-inflammatory activity of nano multiple emulsion was relatively lower than that of nanoemulsion. This observation supports the view that slow pharmacological actions of nano multiple emulsion containing aspirin are produced by the controlled release behavior of encapsulated aspirin drug from the inner aqueous droplets. Lending credence to this view is the ability of nano multiple emulsion to produce modestly lower but significant anti-inflammatory and analgesic effects as compared to reference suspension in carrageenan-induced inflammation model, acetic-acid induced writhing and hot plate assays. It is worth mentioning that improved anti-inflammatory and analgesic activity obtained with the use of lower oral dose of 60 mg/kg in both drug nanocarriers, revealed the possibility that both the aspirin-loaded nanoformulations could be effective in the aforementioned three tests at a much lower dose level.

5. Conclusion

In summary, treatment with novel nanoemulsion and nano multiple emulsion containing aspirin exerts anti-inflammatory and analgesic activity in the above three experimental animal models. This is evidenced by pronounced decrease of paw edema induced by carreegenan as well as significant reduction in the number of writhes and reaction latency respectively in both the acetic acid-induced writhing and hot plate assays. The presence of several inflammatory mediators has been reported to be involved in the carrageenan-induced inflammatory response. The anti-inflammatory properties of these two nanoformulations are further confirmed by significant histopathological changes in the amelioration of inflammatory paw tissues. The results demonstrate that nanoemulsion presented the enhanced inhibitory effect against λ-carrageenan-induced inflammation, chemical and thermal induced nociceptions compared to reference suspension. Despite the mild analgesic effect of nano multiple emulsions, the results for two nociceptive tests obtained for group-treated with such complex emulsion systems in relation to control group were statistically significant. The anti-inflammatory and analgesic properties which were shown in this study, in addition to the aspirin features make these two nanoformulations potential targets for the development of new drug delivery systems that can be explored as alternatives to existing drugs in the current market. Based on the present results, it is strongly suggested that nanoemulsion and nano multiple emulsion generated using ultrasound cavitation technique can be considered as new nanocarriers for pharmacological NSAIDs agent in treating various diseases associated with inflammation and pain. The mechanism involved is not determined and elucidated in the present study and is therefore the likely focus of subsequent study.

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