AJMBR
AlQalam Journal of Medical and Biological Research
AlQalam Journal of Medical and Biological Research,2017;1(1):1-8
LN: 207/2015
Available Online: http://www.utripoli.edu.ly

Screening for Analgesic and Anti-Inflammatory Effect of Antioxidants by Applying Formalin Test Using Albino Mice

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Received:26,06,2017 ***Accepted: 08,07,2017***

ABSTRACT

Objective**:** *Aim of this work is to evaluate analgesic and anti-inflammatory effects of antioxidants.* ***Methods:*** *Formalin test was applied using albino mice. This model constitutes two distinct phases: the first phase represents the irritating effect of formalin at the sensorial fiber-C, while the second phase is inflammatory pain response.* ***Results*****:** *Vitamin C, vitamin E and selenium have analgesic effect for neuropathic pain. Vitamin E and selenium produce anti-inflammatory effect.****Conclusion*****:** *Analgesic effect for neuropathic pain was observed with vitamin C, vitamin E and selenium treatment. Anti-inflammatory effect is produced by vitamin E and selenium administration; while Vitamin C produces insignificant decrease in inflammation in the dose used.*

Keywords: ***Antioxidant, Vitamin C, Vitamin E, Selenium, Formalin Test***.

INTRODUCTION

Antioxidants, such as vitamin C, vitamin E and selenium, are a reducing agent, protect the body from the oxidation reaction that induced by free radicals [1]. They have important roles in the biochemistry of living organisms [2,3]. Antioxidants are either water soluble (hydrophilic) as vitamin C or lipids soluble (hydrophobic) as vitamin E [4]; hydrophilic antioxidant protect cell cytosol and blood plasma from oxidation, while hydrophobic antioxidant protect cell membrane from lipid peroxidation. These antioxidants may be synthesized in the body or obtain as nutrition [5].

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are normally generated either from mitochondrial electron–transport chain or excessive stimulation of NADPH leading to oxidative stress, that damage cell structure including lipid, membrane, protein, and DNA [6].

Cite this article: Aburawi S, Al Tubuly R, Friwan A, Al-Ghdamsi M. Screening for analgesic and anti-inflammatory effect of antioxidants by applying formalin test using albino mice. *Alq, J, Med, Bio Res*, 2017;1(1):1-8

Vitamin C (L-ascorbic acid) is safe and effective antioxidant nutrient. It is needed by the body to make collagen. Vitamin C is used as therapy for scurvy, increase the absorption of iron, and enhance immune function [7]. Vitamin C is used to prevent and treat cold, infection, bronchitis, human immunodeficiency (HIV) disease, stomach ulcer caused by bacteria Helicobacter pylori, tuberculosis, dysentery, and skin infection. Also, it may be used for depression, thinking problems, dementia, physical and mental stress, fatigue and attention deficit-hyperactivity disorder (ADHD) [8].

Vitamin E as a fat-soluble antioxidant, protect cell damage induced by free radical [9]. Vitamin E has a role in immune function, cells - cell signaling, regulation of gene expression and other metabolic processes [10].

Selenium as mineral antioxidant, plays key role in metabolism and protect cell from damage [11]. The aim of this study is to screen for analgesic and anti-inflammatory effect of antioxidants.

Antioxidant used were water soluble vitamin (vitamin C), fat soluble vitamin (vitamin E) and mineral as selenium. Formalin test was applied in this study.

METHODOLOGY

Materials

Tween 80 was purchased from 250mg/kg. Vitamin C was obtained from pharmaceutical chemical company, Elnaser – Egypt. Vitamin E was obtained from Genesisuit company, Doral, Florida, USA. Selenium was obtained from Jamieson company, Toranto - Canada. Tramadol was purchased from Medis BP company, Nabeul - Tunisie, while lysine acetylsalicylate from Amiriya company, Alexandria - Egypt. Formalin was purchased from Al Hashan pharmacy Ain zara- Tripoli- Libya.

Animals

Male albino wister mice weighing between (25-40gm) were housed at constant room temperature (20-25°C) under 12h light/12 dark cycle and with access to food and water with standard mice food pellet diet; water was available *ad libitum*. Mice were housed in the Faculty of Pharmacy, Tripoli University. Each group of mice was housed separately in a cage during the time of experiment.

Methods

Mice were randomly assigned to receive different treatments or vehicle. Tween 80 (1%) was used as suspending agent for all drugs and as a control (Rogoz et al., 2005). A volume of injection of 5ml/kg of body weight was adopted for all experiments (Swayeh et al., 2000).

Mice were divided randomly into six groups of (n=8) animals. Group 1, control group, administered 1% Tween 80, group 2, administered vitamin C (250mg/kg), group 3, administered vitamin E (400UI/kg), group 4, administered selenium (50µg/kg), group 5, administered tramadol (5mg/kg), group 6, administered acetyl salicylic acid (200mg/kg).

Different concentration of drugs was freshly prepared prior to use. Subacute administration was applied, where the drugs injected (i.p.) three doses (24, 5, 1 hour) before injecting formalin and scoring.

Acetyl salicylic acid is used as standard (Guimarães et al., 2009) for formalin test phase II (anti-inflammatory), and tramadol as standard drug for formalin test phase I (analgesic) (Rajani et al, 2009).

Screening of analgesic and anti-inflammatory effect of antioxidants were carried out using formalin test.

Formalin-Induced Nociception

Formalin 3% was injected subcutaneously into the surface of the right hind paw using a 30-gauge needle. The animals were then placed in a clear Plexiglas cylinder (20_30 cm) for observation. The pain behaviour was quantified by determining the duration of time (s) the mouse spent licking the injected paw (Abbot et al, 1995).

Two phases of spontaneous licking behaviour were observed after the formalin injection. The interval from 0 to 5 min has been defined as Phase I (neuropathic pain phase), and the interval from 15 to 30 min has been defined as Phase II (inflammatory pain phase) (Abbot et al, 1995; Guimarães et al., 2009).

Statistical analysis

Descriptive statistical analysis was performed using computer program SPSS (version 13) to verify whether the data were parametric by using Kolmogrove-Simirnove test maximum deviation test for goodness of fit. If the parameters were parametric, treatments were compared by one-way ANOVA, Post-Hoc test (LSD and Duncan test) was applied. If the parameters were non-parametric, treatments were compared by Mann-Whitney U test. The differences were considered significant at P value ≤0.05. The values are expressed as mean ± standard error.

RESULTS

Screening of analgesic effect using formalin test (phase I)

During phase I (analgesic effect for neuropathic pain), Vitamin C, vitamin E, selenium, tramadol, and acetyl salicylic acid showed an increase in the duration of licking at p=0.006, p=0.000, p=0.000, p=0.000, p=0.000 respectively compared to the control (table 1).

Table 1: Screening for analgesic effect of antioxidants applying formalin test (Phase I)

Treatment (n=8)	Duration of licking (Phase I)
1% T80 (control)	113.8 ± 10.64
Vitamin C (250 mg/kg)	78.1 ± 4.63 *

Vitamin E (400 IU/kg)	66.6 ± 10.33 *
Selenium (50 µg/kg)	58.6 ± 12.34 *
Tramadol (5 mg/kg)	55.3 ± 1.87 *
acetyl salicylic acid (200 mg/kg)	63.6 ± 7.35 *

* Significantly different from control at p≤0.05.

Screening of anti-inflammatory effect using formalin test (phase II)

During phase II (anti-inflammatory phase), Vitamin C produce insignificant decrease in the duration of licking (p=0.059); while vitamin E, selenium, tramadol, and acetyl salicylic acid showed an increase in the duration of licking at p=0.000, p=0.000, p=0.000, p=0.000 respectively compared to the control (table 2).

Table 2: Screening for anti-inflammatory effect of Antioxidants applying formalin test (Phase II)

Treatment (n=8)	**Duration of licking (Phase II)**
1% T80 (control)	214.3 ± 60.02
Vitamin C (250 mg/kg)	139.5 ± 23.41
Vitamin E (400 IU/kg)	35.6 ± 8.59 ***,** a
Selenium (50 µg/kg)	39.1 ± 9.71 ***,** a
Tramadol (5 mg/kg)	44.5 ± 9.06 ***,** a
acetyl salicylic acid (200 mg/kg)	60.1 ± 7.84 ***,** a

*, Significantly different from control at p≤0.05; a, Significantly different from vitamin C at p≤0.05

DISCUSSION

Oxidative stress is an important mediator of damage to cell structure including lipid, membrane, protein, and DNA [6]. The body forms ROS endogenously, when it converts food to energy, while antioxidant protect cells from damaging effect of ROS [9].

Reactive oxygen species (ROS) contributes to the development of exaggerated pain hypersensitivity during persistent pain [12]. Formalin test is one of the most used models to explain pain and analgesia mechanisms, with better results than tests using mechanical or thermal stimulus [13]. This model constitutes two distinct phases: the first phase represents the irritating effect of formalin at the sensorial fiber-C, while the second phase is an inflammatory pain response.

Vitamin C exerted its antinociceptive effects primarily because of its antioxidant properties; it has a major role in reducing pain in many clinical conditions [14]. Dopamine receptors agonists facilitate analgesic responses [15,16]. Antinociceptive action of vitamin C was reduced by metoclopramide (dopamine antagonist) [17]. Dopaminergic system involved in the mechanisms of antinociception of vitamin C [18].

Spinal analgesic actions are modulated through 5-HT released from brainstem structures [19-21]. Vitamin C analgesic effect was inhibited by ondansetron, a serotonin antagonist [22]; therefor vitamin C may modulate its analgesic effect through 5HT release.

NMDA receptor, which is essentially contributes to nociceptive processing during both inflammatory and neuropathic pain, is redox regulated [23]; its activity can be inhibited by Vitamin C administration [24]. Therefore, Vitamin C may produce its antinociception by interaction with ionotropic glutamate receptors [12,24].

Three major members of MAPK family (Mitogen-Activated Protein kinase, p38, ERK and JNK) play a major role in the maintenance of persistent pain [25]. Peripheral nerve injury leads to activation of p38, ERK and JNK in glial cells, leading to the synthesis of proinflammatory/pronociceptive mediators, enhancing and prolonging neuropathic pain. Vitamin C treatment reduces p38 but not ERK (p42/p44) phosphorylation in the spinal cord and dorsal root ganglia; this indicates that vitamin C inhibits p38-dependent nociceptive signaling in spinal cord microglia and dorsal root ganglia neurons [26].

Vitamin E produces analgesic effect in phase I. Analgesic effects of vitamin E may be partially explained through its antioxidant properties, involve blocking the production of reactive oxygen species (ROS) that are involved in neuropathic pain. Vitamin E produces analgesia in neuropathic pain that is, mediated by reducing central sensitization [27]; it has the ability to

make the brain less sensitive to pain.

Alpha-tocopherol produces pain relief in patients with algomenorrhea through endogenous opioid system. There was an increase in beta-endorphin after alpha-tocopherol administration and naloxone administration resumed the pain [28].

Vitamin E treatment reduces p38 but not ERK (p42/p44) phosphorylation in the spinal cord and dorsal root ganglia, leading to inhibition of p38-dependent nociceptive signaling [26].

Tramadol produces analgesic effect in phase I. Tramadol hydrochloride is an orally active, centrally acting analgesic [29,30]; it possesses opioid agonist activity and activates the spinal pain inhibitory system [31]. Tramadol produce its action through binding to μ-opioid receptor [32,33].

Tramadol provide pain relief by blockade of serotonin action [34-36], through 5-HT1A auto receptors from raphe nuclei [37]; also, tramadol inhibits the reuptake of serotonin and norepinephrine [32,33].

Selenium produces analgesic effect in phase I, it may produce its effect through increasing the analgesic activity of opioids, as observed using vincristine model of chemotherapeutic-induced painful toxic neuropathy; this model resulted in progressive decrease of pain threshold [38].

Selenium, as an antioxidant, has protective effects on cytosolic Ca2+ release in cell lines; it decreases calcium ion influx and oxidative stress through voltage gated and melastatin-like transient receptor potential 2 (TRPM2) cation channels [40].

Selenium protect from H2O2 that increase Ca2+ influx and oxidative stress through regulation of TRPM2 channels. It was found that, selenium inhibits completely H2O2-induced TRPM2 activation; also, decrease cytosolic Ca2+ release and lipid peroxidation levels [40].

TRPM2 channels involved in nociceptive processing [41,42] and the production of chemokine in inflammation [43]. Therefore, blocking nociception-related TRPM2 channels by selenium will produce analgesic effect [42].

Acetylsalicylic acid produces analgesic effect in phase I, it has antinociceptive activity in formalin test. It significantly increased brain serotonin (5-HT) content and reduced the number of 5-HT2 receptors in cortical brain membranes. It was suggested also that acetylsalicylic acid may exert its antinociceptive action through opiatergic pathways as antinociceptive activity of acetylsalicylic acid is abolished by naloxone [44].

Vitamin C produces insignificant anti-inflammatory effect (phase II); from previous work, free radicals have pro-inflammatory effects [45]; also in clinical trial, vitamin C supplementation for two months resulted in a decrease the level of C-reactive protein (CRP, inflammatory marker) in those with elevated CRP levels [46]; but no effect of vitamin C supplement in those with baseline levels of CRP. Vitamin C significantly reduce pro-inflammatory cytokines (cytokines IL-1α, IL-2, IL-8, TNF-α, chemokine, eotaxin and C-reactive protein) [47]. From previous research, it was found that vitamin C has anti-inflammatory effect; in this study, Vitamin C produces insignificant reduction in inflammation, this may due to vitamin C dose should be increased to have a significant reduction in inflammation.

Vitamin E produces anti-inflammatory effect (phase II), this may due to its antioxidant functions [48]. Vitamin E has anti-inflammatory effect by inhibiting the release of arachidonic acid and the conversion of arachidonic acid to prostaglandin (PGE2) via an action on phospholipase A2 and cyclooxygenase enzymes [49,50].

Alpha-tocopherol exerts its anti-inflammatory effects through a decrease in the levels and inhibits the activity of protein kinase C; also by decreases the level of pro-inflammatory cytokines and inhibits cyclooxygenase-2 [48,52].

Complement-neutrophil-reactive oxygen activation feedback (CNAF) mechanism mediated inflammatory response; it was found that, vitamin E inhibits CNAF mechanism [52]. Vitamin E have been shown to decrease inflammatory prostaglandin synthesis, interleukin production, and the induction of cyclooxygenase-2 (COX-2) and NADPH oxidase by UV light exposed skin [53-55].

Selenium produces anti-inflammatory effect (phase II), complement-neutrophil-reactive oxygen activation feedback (CNAF) mechanism mediated inflammatory response was inhibited by sodium selenite [52]. Selenium suppresses the redox-sensitive transcription factor NF-kappa B-dependent pro-inflammatory gene expression. Selenium increases the production of 15d-PGJ2 as an adaptive response to protect cells against oxidative stress-induced pro-inflammatory gene expression [56].

Selenium plays an important role as an anti-inflammatory agent by tightly regulating the expression of pro-inflammatory genes in immune cells. Selenium anti-inflammatory effect is through a decrease in Lipopolysaccharides-induced expression of two

important pro-inflammatory genes, cyclooxygenase-2 (COX-2) and tumor necrosis factor-alpha (TNF-alpha) and via the inhibition of Mitogen-activated protein kinases (MAPK) pathways [57]; where MAPK stimulates proinflammatory cytokines, while Lipopolysaccharides (LPS) is bacterial endotoxin.

Tramadol produces anti-inflammatory effect (phase II), this may be through reducing prostaglandin E2 concentrations in inflammatory exudates [578,59]. Also through serotonergic and noradrenergic transmission, it may reduce inflammatory edema; tramadol inhibits noradrenaline and serotonin reuptake, leading to analgesic action by blocking nociceptive impulses at the spinal level [60,61]. Tramadol may inhibit inflammatory mediators such as IL-6 in patients and reduce the release of substance p, as observed with knee osteoarthritis [62].

Acetylsalicylic acid produces anti-inflammatory effect (phase II), it inhibited the production of prostaglandins via an inhibitory effect on cyclooxygenase (COX) enzymes; this may account for its anti-inflammatory action [63-65].

Salicylic acid modulates signaling through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), which responsible for cytokine production; it is a transcription factor complex that plays a central role in many biological processes, including inflammation [66].

CONCLUSION

Vitamin C, vitamin E and selenium have analgesic effect for neuropathic pain. Vitamin E and selenium produce anti-inflammatory effect; while Vitamin C produces insignificant decrease in inflammation in the dose used. Vitamin C may show anti-inflammatory effect with increasing the dose.

DISCLOSURE STATEMENT

Conflect of interest statement was not declared.

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