

ORIGINAL ARTICLE
ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ

Gastrointestinal tolerability and efficacy of 2-isopropyl-5-methylphenol in the treatment of osteoarthritis in an animal model

OBJECTIVE To evaluate the gastrointestinal (GI) tolerability and efficacy of 2-isopropyl-5-methylphenol in the treatment of osteoarthritis (OA) in a rabbit model. **METHOD** A study was conducted on 28 healthy male albino rabbits, aged 6–8 months, which were divided into four groups randomly. Group A was a healthy control group; group B was a diseased, untreated; group C and group D were diseased and received paracetamol and 2-isopropyl-5-methylphenol, respectively. The study was divided into two phases. During Phase I, OA was induced in the “diseased” rabbits, using a 4% papain solution (0.2 mL) with cysteine HCl (0.1 mL) as an activator, on the first, fourth and seventh days. Development of disease was confirmed using knee X-ray analysis, estimated by the Kellgren-Lawrence (KL) scale. During Phase II, the animals in groups C and D were treated with paracetamol and 2-isopropyl-5-methylphenol, respectively. Knee X-rays were taken at 4-week intervals and blood tests were made before and at the end of treatment, including complete blood count, renal function tests and liver function tests. The pain score was calculated using the Rabbit Grimace Scale (RGS). **RESULTS** The OA pain disappeared after day 4 in the groups treated for pain alleviation. Knee X-ray showed that OA was reversed and returned to KL score “0” in group D. The 2-isopropyl-5-methylphenol treatment considerably decreased the blood levels of alanine aminotransferase, aspartate aminotransferase and alkaline phosphate ($p=0.001$), and improved renal and liver function. **CONCLUSIONS** 2-isopropyl-5-methylphenol is safe and effective in the control of pain and the inhibition and reversal of OA, and is tolerated by the GI tract in the experimentally induced rabbit model.

Osteoarthritis (OA) is a disorder involving movable joints, characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses, including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement, followed by anatomic, and or physiological derangements, which can culminate in illness (OARSCI). Approximately 250 million people worldwide have knee osteoarthritis, or 3.6% of the population.¹ A study on the prevalence of knee osteoarthritis in Pakistan showed that 28% of the urban and 25% of the rural population had osteoarthritis.²

The primary causes of osteoarthritis are injury, obesity, aging and genetics, while secondary causes include alkapa-

tonuria, congenital disorders of the joints, hemochromatosis, Wilson’s disease, injury to joints or ligaments, obesity, joint infection, inflammatory disease, and diabetes mellitus.³

Various pathophysiological mechanisms and etiological risk factors contribute to the progression of the disease and aid as markers of the disease and response to pharmacological intervention. The pathophysiology affects all three joint tissue partitions and is characterized by osteophyte formation, breakdown of articular cartilage, bone marrow lesions, subchondral sclerosis and modifications of the synovium at both the biochemical and morphological stages, causing occasional synovitis. The cytokine-based molecular events that contribute to joint injury in inflammatory arthritis have progressively appeared as pathogenic

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ΑΡΧΕΙΑ ΕΛΛΗΝΙΚΗΣ ΙΑΤΡΙΚΗΣ 2017, 34(6):790–796

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Ανεκτικότητα από το γαστρεντερικό και αποτελεσματικότητα της 2-ισοπροπυλ-5-μεθυλφαινόλης στη θεραπεία της οστεοαρθρίτιδας σε πειραματόζωα

Περίληψη στο τέλος του άρθρου

Key words

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patterns in OA and will be highly pertinent to the advancement of novel OA treatment.

The diagnosis of OA is based on the history, clinical examination and X-ray appearance. A number of classification systems are used for the gradation of OA, including the Western Ontario and McMaster Universities Arthritis Index (WOMAC) score and the Kellgren-Lawrence (KL) grading system, which is a scoring tool used to assess the severity of knee OA on a plain X-ray.⁴

Lifestyle modification, including weight loss and exercise, and patient education are good in osteoarthritis management. Acetaminophen, as an analgesic, is the first line treatment for OA on a short-term basis. Non-steroidal anti-inflammatory drugs (NSAIDs) are more effective for mild to moderate symptoms. Oral opioids such as tramadol are also often prescribed, but are recommended only when first line therapy has failed or is contraindicated. When problems persist and more conservative management is not beneficial, joint-replacement surgery or joint resurfacing may be recommended. There is little or insufficient evidence to support the benefits claimed to be associated with certain supplements, including the Ayurvedic herbal preparations, collagen, ginger, glucosamine, hyaluronic acid, etc. Thymol (2-isopropyl-5-methylphenol) is a natural monoterpene phenol derivative of cymene, C₁₀H₁₄O, isomeric with carvacrol. It has been observed that thymol is a major component of many plants, including *Trachyspermum ammi* (Ajwain). It is reported that Ajwain oil contains thymol (39.1%) as a major component, along with p-cymene (30.8%), γ -terpinene (23.2%), β -pinene (1.7%), and terpinene-4-ol (0.8%) (5). The objective of this study was to investigate the gastrointestinal (GI) tolerability and the efficacy of 2-isopropyl-5-methylphenol for pain relief in the treatment of OA in a rabbit model.

MATERIAL AND METHOD

Animals

The study was carried out on 28 albino male rabbits aged 9 to 12 months. The animals were divided into four groups of 7, randomly, by the flip of coin method. Group A was the control group, group B was the diseased, untreated group, group C and group D were diseased and received paracetamol and 2-isopropyl-5-methylphenol, respectively. The animals were kept under controlled conditions of temperature 23±2 °C, at a 12-hourly light and dark cycle, and were given food and water *ad libitum*.

Plant material and preparation of extract

The seeds of *T. ammi* L. were purchased from a local herbal

store in Lahore. The plants were identified and authenticated by the Department of Botany, University of the Punjab, Lahore. The seeds were rendered free of all impurities manually and were soaked in methanol for 30 days. The soaked material was then filtered through filter paper and the filtrate was passed through a rotary evaporator and then freeze dried. Further extraction of 2-isopropyl-5-methylphenol was performed according to the method described by Rajput et al.⁴

Instrumentation

Rabbit grimace scale: The pain score was calculated using the Rabbit Grimace Scale (RGS). An RGS score for each radiograph was calculated by averaging intensity ratings for each action unit (AU). The AUs include orbital tightening, nose shape, cheek flattening, whisker change, and ear position. No pain was scored as 0, moderate pain as 1 and severe pain as 2. An RGS difference score (relative to baseline/no pain) was calculated for each animal, and averaged across a group.

Radiographic techniques: Antero-posterior X-rays of the knee joint were obtained, using standard projections. Global joint assessment was made according to the KL scale, as explained in the Atlas of standard radiographs of arthritis,⁵ classified as grade 2 or higher defined OA. An experienced radiologist scored each X-ray for the presence and severity of OA using the KL scale.

Biochemical tests: Blood was drawn from the marginal ear vein of the rabbits. Liver function tests, renal function tests and complete blood count were performed.

Methods

Before induction of disease, biochemical tests and X-ray analysis were performed. The study was divided into two phases. During Phase I, OA was induced experimentally by methods specified by Bentley (1971), using 4% papain solution (0.2 mL) and cysteine HCl (0.1 mL) as an activator on the first, fourth and seventh days. The development of the disease was confirmed using X-ray radiographic imaging. During Phase II, the animals were treated according to the group. Group A was the control, group B was diseased, untreated for pain relief, group C received 200 mg paracetamol BD, and group D received 2-isopropyl-5-methylphenol 5 mg BD. Knee X-rays were made at four-week intervals and the pain score calculated using the RGS. The biochemical tests were repeated before and at the end of treatment.

To assess the GI tolerability and safety of the treatment protocols, various biochemical tests were performed, comprising liver function tests, renal function tests and complete blood count. The liver function tests included serum bilirubin (s. bilirubin), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). The renal function tests included blood urea nitrogen (BUN), blood urea, serum creatinine (s. creatinine) and serum uric acid (sUA). The complete blood count included hemoglobin (Hb), white blood cell count (WBCs), red blood cor-

puscle count (RBCs), platelet count (PLT), neutrophil count (Neut), lymphocyte count (Lym), monocyte count (Mon), eosinophil count (Eosin), basophil count (Baso), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

Statistical analysis

Statistical analysis was performed with the Statistical Package for Social Sciences (IBM SPSS), version 22.0 and included one way ANOVA and *post hoc* tests (Tukey test). The value 0.05 was considered as significant.

Ethics

The research protocol was approved by the Animal Ethical Committee of Hajvery University, Lahore, Pakistan.

RESULTS

Efficacy assessment

Signs of pain were observed in the rabbits on induction of OA from day 0 to day 4. Pain was severe on day 1 and reduced over the next few days. A little pain was found at day 4. According to the RGS scoring system, the most severe pain was observed in group B (diseased), with a mean pain score of 0.43 (fig. 1). A similar pain scenario was observed in groups C and D on day 1, with higher pain intensity on day 1 than on day 0. On day 2, the pain score was higher in group B than in group C (paracetamol) and

group D (2-isopropyl-5-methylphenol). On day 3 the pain was significantly reduced in group D in comparison with other groups. Pain was not observed on day 4 in most treated animals, although some still demonstrated a little pain. Consequently, it was observed that group D treatment was more effective in reducing pain than group C treatment or no treatment (fig. 1).

The antero-posterior X-rays showed that bilateral OA was induced in the rabbits' knees by the OA induction process. According to the KL grading system, usually grade 3 OA developed in the right knee and grade 4 in the left knee, although this varied within groups and between knees. After 4 weeks of treatment, OA disease progression was reduced to KL grade 2 in group D. After 8 weeks of treatment, in group D the signs of OA were reversed and had returned to KL grade 0, in group C the disease was KL grade 2, while in group B which was untreated, the KL score remained at grade 4 (figures 2, 3).

Safety assessment

After treatment, the liver enzymes levels were significantly lower in group D than in the other diseased groups ($p=0.001$) (fig. 4). A significant increase was observed in the liver enzymes levels in group C, indicating possible toxicity.

The BUN and urea levels were lower in group C than in D ($p=0.005$ and $p=0.001$, respectively), but the s. creatinine and sUA levels were higher ($p=0.001$) (fig. 5). It was observed that group D had a comparatively balanced blood count, compared with the other diseased groups (tab. 1).

Gastrointestinal tolerability

Healthy rabbits produce two types of feces. Hard round feces of intestinal origin are rich in small pieces of hay and other debris. They can be seen around or in the litter-box. Smelly, soft, grape-like cecotropes (also called soft cecal pellets, coated with a thin layer of mucus) are produced in the cecum. They are rich in minerals, vitamins, proteins, water, and bacteria. Any disturbance of the intestinal environment can lead to a change in the fecal shape. The feces will be small and dry when the rabbit is dehydrated or sick, or large and elongated when there is lack of fiber in the diet. The rabbits in the study produced cecal pellets.

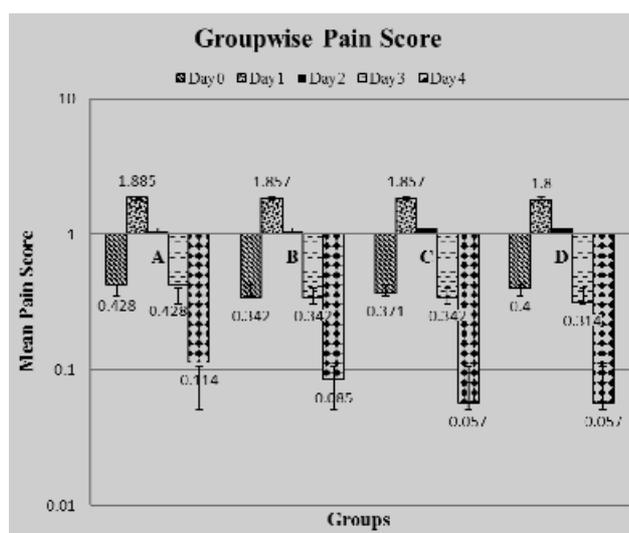


Figure 1. Pain score in the four groups of animals; A, B, C, D. A: Control group, B: Osteoarthritis (OA) group, no treatment, C: OA group, treated with paracetamol, D: OA group, treated with 2-isopropyl-5-methylphenol.

DISCUSSION

The pain caused by induced OA was severe on day 1 from the start of treatment and diminished after day 4, as



Figure 2. Comparison of right knee X-rays of rabbits after 8 weeks of treatment. A: Control group, B: Osteoarthritis (OA) group, no treatment, C: OA group, treated with paracetamol, D: OA group, treated with 2-isopropyl-5-methylphenol.



Figure 3. Comparison of X-rays of left knees of rabbits after 8 weeks of treatment. A: Control group, B: Osteoarthritis (OA) group, no treatment, C: OA group, treated with paracetamol, D: OA group, treated with 2-isopropyl-5-methylphenol.

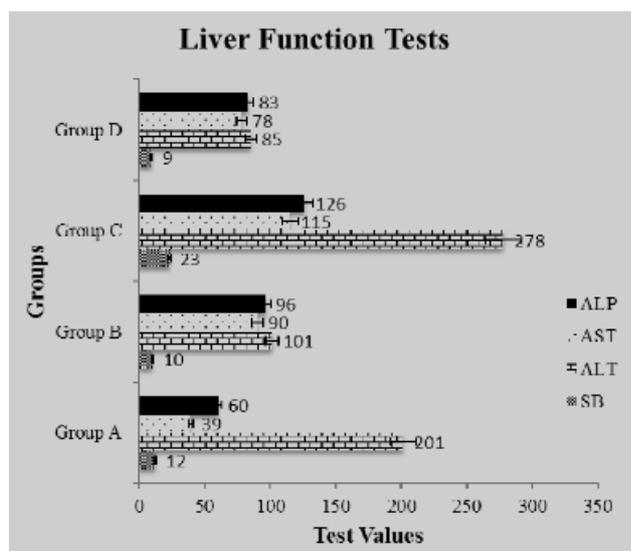


Figure 4. Liver function tests in the four groups of rabbits at the end of treatment. A: Control group, B: OA group, no treatment, C: OA group, treated with paracetamol, D: OA group, treated with 2-isopropyl-5-methylphenol, ALP: Alkaline phosphatase, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, SB: Serum bilirubin.

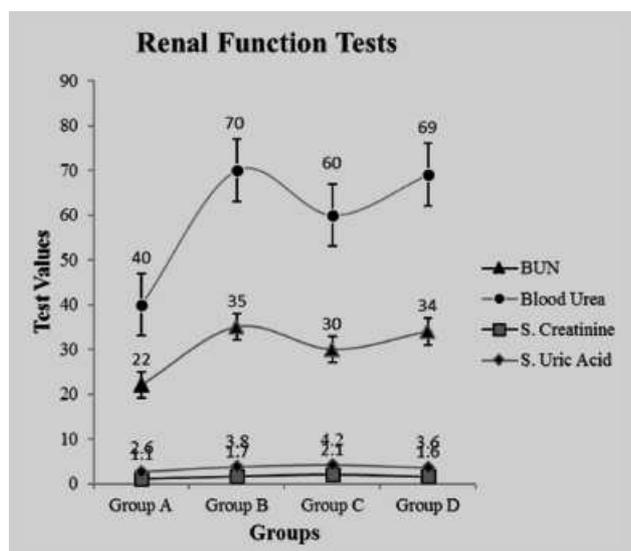


Figure 5. Renal function tests in the four groups of rabbits at the end of treatment. A: Control group, B: Osteoarthritis (OA) group, no treatment, C: OA group, treated with paracetamol, D: OA group, treated with 2-isopropyl-5-methylphenol, BUN: Blood urea nitrogen.

shown in figure 1. The pain was reduced significantly more in group D. These findings parallel those in the study of Rajput and colleagues,⁶ who concluded that postoperative pain in mice lasted for 36 to 48 hours, and appeared to show relative exacerbation during the early dark (active) photo-

Table 1. Complete blood count in the four groups of rabbits at the end of treatment.

Tests	Group A	Group B	Group C	Group D	Normal values
Hb (g/dL)	11	10.7	6.9	10.4	9.4–17.4
WBC (10 ⁹ /L)	6	7.4	2.1	3.2	5.0–13.0
RBC (10 ⁶ /mm ³)	4.1	5.07	3.45	5.34	3.8–7.9
PLT (g/dL)	196	152	86	158	193–725
Neut (%)	51	37	41	45	34–70
Lym (%)	56	60	50	50	43–80
Mon (%)	1	2	5	3	00–04
Eosin (%)	1	1	4	2	00–02
Baso (%)	0	0	0	0	00–0.84
MCV (mm ³)	61	53.9	63.6	58.31	50.0–75.0
MCH (Pg)	22	21.1	20	19.4	18.0–24.0
MCHC (%)	32	39.1	31.5	33.4	27.0–34.0

A: Control group, B: Osteoarthritis (OA) group, no treatment, C: OA group, treated with paracetamol, D: OA group, treated with 2-isopropyl-5-methylphenol.

phase. Consequently, it is concluded from the findings of the present study that 2-isopropyl-5-methylphenol is useful in reducing OA pain. The efficacy of the treatment was assessed by knee X-rays at the start of treatment, and after 4 and 8 weeks of treatment, using the KL grading scale.⁵ According to the KL grading system, grade 3 OA was induced in the right knee and grade 4 in the left knee, with slight variation. After 4 weeks of treatment, the X-rays showed that the OA progression was reduced to KL grade 2 in group D, and by 8 weeks to grade 0, showing complete reversal of the OA. In group C disease progression had reversed to KL grade 2 after 8 weeks of treatment. A randomized, double blind, placebo controlled, crossover study was carried out to assess the safety, efficacy and tolerability of *Boswellia serrata* extract (BSE) in knee OA. All drug treatment groups reported lowering of knee pain, increased walking distance and improved knee flexion, and the frequency of knee joint swelling was decreased, but with no radiological change. BSE was well tolerated by the patients except for minor GI adverse events. BSE is therefore recommended for knee OA, with possible therapeutic use in other forms of arthritis.⁷

To assess the safety of the treatment protocols under investigation, various biochemical tests were also performed. Liver function tests are routinely used to estimate liver disease severity, toxicity and hepatic dysfunction.⁸ All the liver enzyme levels were significantly lower in group D animals at the end of treatment than in the other diseased groups. In an equivalence study comparing topical diclofenac solution with oral diclofenac in the symptomatic

treatment of knee OA, Tugwell and colleagues found that the oral diclofenac group developed abnormal liver function tests, hemoglobin, and creatinine clearance.⁹ A significant increase was observed in the liver enzymes level in group C, indicating toxicity. Thus, in relation to other studies on OA, the current study showed that 2-isopropyl-5-methylphenol is the most hepatoprotective of the treatments studied.

The renal function tests investigated include BUN, blood urea, s. creatinine and sUA. It was observed that BUN levels were statistically non-significant between group C and D. However, there was a significant difference in the levels of blood urea, s. creatinine and sUA between the treatment groups, as depicted in figure 4, but the levels in group D were no different from the untreated OA group, indicat-

ing that 2-isopropyl-5-methylphenol is not nephrotoxic. The complete blood count is a parameter that has been used in other studies for exploring the safety of treatment protocols.^{10,11} The results of the present study indicate that 2-isopropyl-5-methylphenol had no adverse effect on the complete blood count on the animals in group D, as compared to paracetamol.

In conclusion, this study was conducted to determine the GI tolerability, safety and efficacy of 2-isopropyl-5-methylphenol in the treatment of OA in a rabbit model. This study revealed that 2-isopropyl-5-methylphenol is a safe and effective agent in the inhibition and reversal of OA in an experimentally induced rabbit model. It is the first agent found harmless for the GI tract.

ΠΕΡΙΛΗΨΗ

Ανεκτικότητα από το γαστρεντερικό και αποτελεσματικότητα της 2-ισοπροπυλ-5-μεθυλφαινόλης στη θεραπεία της οστεοαρθρίτιδας σε πειραματόζωα

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ΣΚΟΠΟΣ Η εκτίμηση της γαστρεντερικής ανεκτικότητας και της αποτελεσματικότητας της 2-ισοπροπυλ-5-μεθυλφαινόλης στη θεραπεία της οστεοαρθρίτιδας σε μοντέλα κουνελιού. **ΥΛΙΚΟ-ΜΕΘΟΔΟΣ** Είκοσι οκτώ άρρενα υγιή αλφικά κουνέλια, ηλικίας 6–8 μηνών, διαχωρίστηκαν σε τέσσερις ομάδες τυχαία μετά από επαγωγή της νόσου, εφαρμόζοντας τη μέθοδο του κέρματος. Η ομάδα Α συνιστούσε την ομάδα ελέγχου, η ομάδα Β νοσούσε, οι ομάδες Γ και Δ λάμβαναν παρακεταμόλη και 2-ισοπροπυλ-5-μεθυλφαινόλη, αντίστοιχα. Η μελέτη χωρίστηκε σε δύο φάσεις. Κατά τη διάρκεια της Φάσης Ι, προκλήθηκε οστεοαρθρίτιδα μετά από χορήγηση 4% διαλύματος παπαΐνης (0,2 mL) με υδροχλωρική (HCL) κυστεΐνη (0,1 mL) ως ενεργοποιητή, τις ημέρες 1, 4 και 7. Η ανάπτυξη της νόσου επιβεβαιώθηκε περαιτέρω με ακτινογραφική ανάλυση. Κατά τη διάρκεια της Φάσης ΙΙ, στα υποκείμενα χορηγήθηκαν παρακεταμόλη και 2-ισοπροπυλ-5-μεθυλφαινόλη. Ακτινογραφίες πραγματοποιούνταν κάθε 4 εβδομάδες και βιοχημικός έλεγχος στην αρχή και στο πέρας κάθε θεραπείας, αντίστοιχα. Οι βιοχημικές εξετάσεις περιλάμβαναν γενική αίματος, καθώς και έλεγχο νεφρικής και ηπατικής λειτουργίας. Η ένταση του πόνου υπολογίστηκε χρησιμοποιώντας την κονίκλειο κλίμακα μορφασμού (Rabbit Grimace Scale). Όλα τα δεδομένα αναλύθηκαν με το λογισμικό πρόγραμμα Statistical Package for Social Sciences (SPSS), έκδοση 22.0, χρησιμοποιώντας τη μέθοδο ANOVA. **ΑΠΟΤΕΛΕΣΜΑΤΑ** Ο πόνος εξαφανίστηκε μετά από 4 ημέρες σε όλες τις ομάδες. Οι ακτινογραφίες έδειξαν ότι η οστεοαρθρίτιδα είχε υποχωρήσει και επέστρεψε στο «0» με βάση την κλίμακα Kellgren-Lawrence στην ομάδα Δ. Η 2-ισοπροπυλ-5-μεθυλφαινόλη μείωσε σημαντικά τα επίπεδα της αλανινικής και της ασπαρτικής αμινοτρανσφεράσης, καθώς και της αλκαλικής φωσφατάσης ($p < 0,001$) και βελτίωσε τη νεφρική και την ηπατική λειτουργία. **ΣΥΜΠΕΡΑΣΜΑΤΑ** Η 2-ισοπροπυλ-5-μεθυλφαινόλη είναι αποτελεσματική και καλά ανεκτή από το γαστρεντερικό σύστημα. Επί πλέον, είναι περισσότερο ασφαλής και αποτελεσματική στην αναστολή και στην αναστροφή της οστεοαρθρίτιδας σε πειραματικά μοντέλα κουνελιού. Η παρούσα μελέτη θα πρέπει πιθανόν να εφαρμοστεί και στον άνθρωπο.

Λέξεις ευρητηρίου: 2-ισοπροπυλ-5-μεθυλφαινόλη, Κουνέλι, Οστεοαρθρίτιδα, Παρακεταμόλη, Πόνος

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