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Research Article

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Prevention of post-surgical adhesions by Achnil in New Zealand white Rabbits

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ABSTRACT

Adhesion is an inflammatory repair process that occurs following tissue injury. NSAIDs with their COX inhibition potential are known to prevent adhesions to a certain extent. Their role in thwarting the inflammatory process is responsible for a setback in the repair process ultimately leading to the prevention of adhesions. The present investigation was carried out to prevent post surgical abdominal adhesion by Achnil and diclofenac sodium. 15 female rabbits were randomly assigned to 3 groups containing 5 animals each on the basis of body weight for induction of surgical adhesion and respective treatment. Observations revealed that animals in the control group were weak and emaciated following surgery. Diarrhoea and loss of body weight was evident. Animals in diclofenac sodium group showed minor advantage over control. However, Achnil treatment was able to salvage animals from distress as evidenced by clinical sign data on day-14. No mortality was observed with Achnil treated groups. Oxidative stress and inflammatory parameters like (IL-17 and TNF-α) were improved significantly in the Achnil treated groups as compared to diclofenac sodium. Gross necropsy revealed presence of severe adhesions in the control and diclofenac sodium treated groups. In case of Achnil, adhesions were found to be absent. Adhesion percentage and tenacity were significantly higher in the control groups. diclofenac sodium caused only a modest reduction in the adhesion percentage and tenacity as compared to the Achnil treated groups. Histopathological evaluation showed inflammatory infiltration and collagen deposition in the adhesion tissue on a severe scale in the control group. Inflammatory infiltration was comparable in the Achnil and diclofenac sodium treated groups. Tissue from Achnil treated animals showed only minor infiltration and collagen deposition as compared to control. Based on the results, it was concluded that Achnil is able to prevent surgical adhesions in rabbits.

Key words: aceclofenac; achnil; adhesion; diclofenac; inflammation

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INTRODUCTION

One of the very common complications of abdominal surgery is the development of abdominal adhesions [1-2]. Adhesion refers to the development of scar tissue between the abdominal peritoneal lining and intestinal walls. Such kind of adhesion can also develop between the walls of intestine. Adhesion may be considered as an outcome of the inflammatory process that occurs as a result of surgery or the cause for which surgery was instituted. Scar tissue formation is part of the healing process as a result of the inflammation [1]. It has been documented that more than 50% of abdominal surgeries involving laparotomy are prone to the development of adhesions. Formation of

adhesions are secondary to mechanical damage to the tissues during surgery, microbial infections, ischemia, or bleeding during surgery. Improved and asceptic surgical techniques like laparoscopic procedures modestly reduce the incidence of adhesion [1-3]. To support this practice application of adjuvants is practiced which can drastically reduce the occurrence of adhesions. Adjuvants may vary from mechanical separating films to the use of pharmacological agents like NSAIDs and TNF-α antagonists. NSAIDs in particular have been shown to reduce the development of intra-abdominal adhesions [4-5]. Though concrete mechanisms may not be known for this effect but may be due to the involvement of prostaglandins in the development of adhesions [6]. It is their role in thwarting the inflammatory process which might be responsible for a setback in the repair process ultimately leading to the prevention of adhesions. Aceclofenac and diclofenac are *pan*-COX inhibitors and are reported to prevent cell-adhesions [7-8]. Different studies have shown the preventive role of diclofenac and aceclofenac in adhesions occurring in platelets or neutrophils.

Achnil is a controlled release formulation of aceclofenac. It is indicated for the management of different painful etiologies. It has been shown to exhibit superior analgesic activity in the rat formalin test as compared to other marketed pain relief formulations [9]. It is known to have faster onset and prolonged duration of action as compared to other NSAID preparations with lesser propensity to cause GI-related side effects. The present study was undertaken to evaluate the comparative efficacy of two parenteral preparations, Achnil and diclofenac sodium (Diclofenac) respectively, in an animal model of intra-abdominal adhesions.

EXPERIMENTAL SECTION

2.1 Chemicals

Achnil, diclofenac sodium, ketamine, xylazine, buprenorphine and sutures were purchased locally. Epinephrine, Ellman's reagent (DTNB) and thiobarbituric acid were purchased from HiMedia (Mumbai, India). Enzyme immunoassay kits were procured from Invitrogen, Life Technologies, USA. Other reagents and chemicals were of AR grade.

2.2 Animals

The study was conducted on healthy adult female rabbits (New Zealand White, 2.0-3.0 kg). Animals were procured from licensed animal breeders and maintained on commercially available rabbit chow. Food and drinking water were provided *ad libitum* unless mentioned otherwise. Housing of animals involved a 12 h light/dark cycle at 20±2°C and 30-70% RH. All experimental protocols were approved by the institutional animal ethics committee and all procedures performed were in accordance to the principles of CPCSEA.

2.3 Study Design

15 healthy female rabbits were used for the study. Animals were randomly assigned into 3 different groups (n=5 for each group). Surgery was performed on Day-0 for all animals. Animals were treated for 14 days. The allocation is shown in Table 1 below:

Table 1: Grouping and treatment allocation

Group	Number of Animals	Group	Treatment	Dose, volume & frequency
G-1	5	Control	Surgery + normal saline	1ml/kg; b.i.d.
G-2	5	Achnil	Surgery + Achnil	7.75 mg/kg; 1 ml/kg; s.i.d.
G-3	5	Diclofenac	Surgery+ Diclofenac	3.875 mg/kg; 1 ml/kg; b.i.d.

2.4 Description of Procedure

Surgical adhesion were induced in animals by the rabbit sidewall defect-cecum abrasion procedure described previously [10]. All surgical procedures were carried out under asceptic technique. Anesthesia was induced by intra-muscular injection of ketamine and xylazine (35 mg/kg and 5 mg/kg respectively). To induce adhesions, a 10-cm long incision was made along the linea alba through the periotneum. Subsequently, a 3 ⋅4 cm defect was made in the parietal peritoneum on the right lateral abdominal wall, 1 cm away from the incision. Next the caecum was abraded distal to the ileocaecal junction between the 6^{th} and 12^{th} haustra. Abrasion was performed using a surgical brush and rubbed in the said region for 80-120 times until a bleeding surface was observed. Bleeding was not checked and allowed to heal spontaneously. The incision was closed using vicryl (ethicon®) sutures. Buprenorphine (0.05 mg/kg;

s.c.) was administered b.i.d. for two days post-surgery. Required doses of diclofenac and Achnil were calculated as per the individual rabbits body weight and the appropriate volume was administered intra-muscularly. Study design is shown in Table 2 below:

Table 2: Study design for surgical adhesion study

Randomization and surgical induction of adhesion	Treatment	Body weight	Body temperature, clinical signs mortality	Phlebotomy, necropsy, adhesion scoring and tissue harvesting
Day-0	Days 1-14	Days-0, 7, 14	Daily	Day 14

2.5 OBSERVATIONS

2.5.1 Body Weight

Body weight of all the animals were evaluated on a weekly basis.

2.5.2 Body temperature

Body temperature of all the animals were evaluated on a daily basis using a rectal probe.

2.5.3 Clinical Signs And Mortality

All the animals were monitored at regular intervals for the first 24 hrs after surgery and daily once thereafter. All animals were observed daily for mortality/morbidity during the entire observation period. Animals found in a moribund condition and animals showing severe pain and enduring signs of severe distress were sacrificed by overdose of ketamine and subjected to detailed necropsy.

2.5.4 Serum estimations

Blood collected was allowed to clot at room temperature. The samples were then spun at 4000 rpm for 10 minutes and serum was collected as the supernatant. Parameters like SOD, reduced glutathione, MDA, Interleukin-17 and TNF-α were determined in the serum.

2.5.5 Gross Necropsy

All the animals were subjected to sacrifice and gross necropsy on the terminal day of the study (14^{th} Day). The pathologist performing the gross necropsy was responsible for recording Adhesion percentage scale (APS, Table 3) and Adhesion tissue tenacity scale (ATTS, Table 4) as per the scoring tables and was blinded to the treatments.

Table 3: Adhesion Percentage Scale

Observation	Score
No adhesion	0
Less than 25% of the abraded area	1
Adhesion in 25-50% area	2
Adhesion in 51-75% area	3
Adhesion in 76-100% area	4

Table 4: Adhesion Tissue Tenacity Score

Observation	Score
No adhesion	0
Tissues separable by gravity	1
Tissues separable by blunt dissection	2
Tissues separable by blunt dissection	3

2.5.6 Histopathology

The Neo-tissue Formed Was Dissected And Harvested In Buffered Formalin For Histopathological Evaluation. Histopathological Evaluation Was Performed After Staining The Sections With Hematoxylin And Eosin And Masson Trichome. Inflammatory Cell Density Was Calculated From Fields of Hematoxylin And Eosin Stained Slides.

RESULTS AND DISCUSSION

Adhesion is one of the inflammatory repair processes that occurs following any tissue injury. One common complication of surgical procedures is the development of anomalous adhesions which can make future surgeries extremely complicated or even impossible [11]. Clinical reports suggest that more than 50% of laparotomy related procedures are known to show adhesion [1-2]. Adhesion may be the result of mechanical damage to the tissues during surgery, transient ischemia or infections [1-3]. Modifications in the surgical techniques can reduce but not altogether prevent adhesions and hence adjuvants are frequently considered for preventing such post-surgical complications. NSAIDs with their COX inhibition potential are known to prevent adhesions to a certain extent [12]. Aceclofenac and diclofenac are *pan*-COX inhibitors and are reported to prevent cell-adhesions [7-8]. The present study was undertaken with the objective of understanding the efficacy of Achnil, a novel formulation of aceclofenac, in preventing intra-abdominal adhesions and also to compare it with an injectable preparation of diclofenac sodium. The cecal abrasion model was chosen for the evaluation of intra-abdominal adhesions since several studies in rodents and rabbits have reported this model to be reproducible and allows useful interpretation of data regarding prevention of adhesion with different interventions [10, 13-15]. The duration of treatments with Achnil and diclofenac were chosen to be for 14 days so as to determine the effect of these preparations. The results are shown below:

3.1 Body Weight

All the animals showed normal body weight gain. However, the animals in the control group showed relatively lesser gain in body weight (Figure 1).

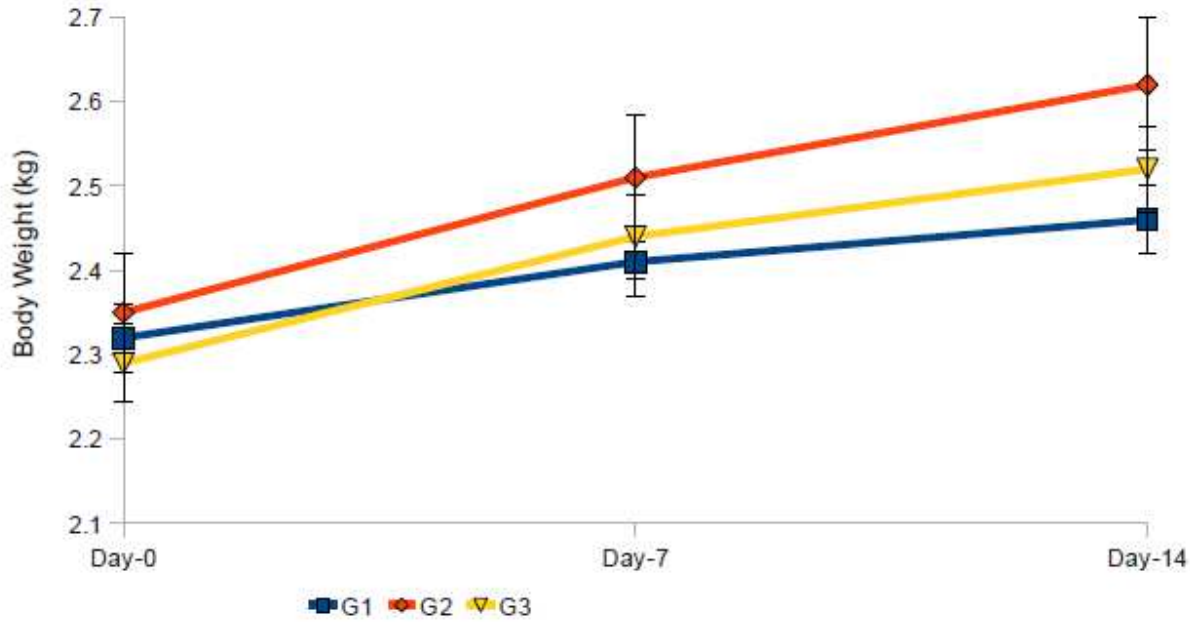

Figure 1: Body weight of animals. Data is presented as Mean ± SEM

3.2 Body Temperature

All the animals showed normal body temperature in the range of 37±2°C.

3.3 Clinical Signs & Mortality

The clinical signs observed in all the groups are summarised in Table 5. It was observed that some animals showed signs of pain, such as increase in lachrymal secretion, reduced appetite, weakness and emaciation. Some animals showed diarrhea and absence of normal weight gain. Symptoms of pain and weakness exhibited by the animals of the disease control group may be a result of the inflammatory repair process. Treatment with Achnil was able to mitigate such a condition whereas diclofenac was found to be less efficient in this regard. Post-operative adhesions are known to cause about 10-20% mortality in cases of laparotomy [16] but no mortality was observed in the present

study.

Table 5: Summary of clinical signs and mortality data recorded for all the groups

Control	Achnil	Diclofenac
G1	**G2**	**G3**
Diarrhea (2/5)*, increase in lacrymal secretion (3/5), decrease in appetite (1/5), dehydrated (2/5)	Diarrhoea (1/5)	Diarrhoea (2/5), prostration (1/5), dehydrated (2/5)
**Figures in the brackets indicate the number of animals from the group showing the respective clinical signs*		

3.4 Estimation of oxidative stress-related parameters in the serum

Surgery and inflammatory process mediated oxidative stress was evident in the animals as disease control animals suggested that MDA levels were higher and SOD as well as GSH levels were found to be lower. Achnil and diclofenac were able to salvage this status, however, it was observed that Achnil mediated a significant positive effect on the oxidative stress in the animals leading to a profound and statistically evident reduction of MDA levels and elevation of SOD & GSH levels.

3.4.1 Malondialdehyde levels

It was observed that malondialdehyde levels were drastically high in the control group. Modest reduction was observed with diclofenac therapy which was not found to be significant at the end of 14 days of study. In contrast, Achnil treated groups exhibited significant reduction ($P<0.05$) in the the elevation of MDA levels in the serum indicating that Achnil treatment reduced oxidative stress in the animals (Table 6).

Table 6: Malondialdehyde levels as measure of lipid peroxidation

Groups	Control	Achnil	Diclofenac
MDA Levels	**G1**	**G2**	**G3**
	1.77 ± 0.13	1.11 ± 0.10*	1.41 ± 0.09
*Data is presented as mean ±SEM. Unit: nmol/mg of protein. * indicate P<0.01 as compared to day-matched control groups*			

3.4.2 Superoxide dismutase (SOD) levels

It was observed that SOD levels in the control groups were found to be lower than normal physiological values suggesting a decline in the antioxidant status of the control animals. Therapeutic intervention was able to rescue this decline to a certain extent and Achnil was found to be superior as compared to diclofenac in the preventing the fall of SOD levels (Table 7).

Table 7: Superoxide dismutase levels from all groups

Group	Control	Achnil	Diclofenac
SOD Levels	**G1**	**G2**	**G3**
	3.07 ± 0.23	3.71 ± 0.10*	3.21 ± 0.29
*Data is presented as mean ±SEM. Unit: IU/mg of protein. * indicate P<0.05 as compared to day-matched control group*			

3.4.3 Reduced Glutathione (GSH) levels

GSH levels indicate the oxidative free radical neutralizing capacity. In the present study, it was observed that GSH levels in the treated groups were higher as compared to the cecal abrasion groups (G2 and G3). Data indicated that Achnil was superior to diclofenac in retaining the higher levels of GSH as compared to control (Table 8).

Table 8: Reduced glutathione levels from all groups

Groups	Control	Achnil	Diclofenac
GSH Levels	**G1**	**G2**	**G3**
	25.3 ± 3.31	36.17 ± 3.4*	32.11 ± 2.9
*Data is presented as mean ±SEM. Unit: mg/ml of serum. * indicate P<0.05 as compared to control group*			

3.5 Estimation of inflammatory parameters in the serum

IL-17 is a pro-inflammatory cytokine responsible for mediating several processes of inflammation including

expression of adhesion molecules like VCAM and ICAM. IL-17 is involved in immune regulatory functions of the mammalian bodies. It is mainly involved in the maintenance of the inflammatory process which forms an intricate part of the healing process. It has also been known to induce post-operative intra-abdominal adhesions [17-18].

3.5.1 Interleukin-17 (IL-17) levels

Significant difference was observed between the Achnil treated and control groups (P<0.05) in the IL-17 levels suggesting that Achnil affects the process of healing and ultimately might reduce adhesions. Diclofenac also affected a modest decline in the IL-17 levels but the change was not found to be statistically significant (Table 9).

Table 9: Interleukin-17 levels from all groups

Groups	**Control**	**Achnil**	**Diclofenac**
IL-17 Levels	**G1**	**G2**	**G3**
	65.3 ± 12.1	32.1 ± 13.4*	52.11 ± 14.7
*Data is presented as mean ±SEM. Unit: pg/ml of serum. * indicates P<0.05 as compared to control group*			

In the present study, it was observed that IL-17 levels were significantly higher in the disease control group suggesting an inflammatory process at work. This aspect was further put to confirmation by estimation of TNF-α levels in the serum. In fact, TNF-α has been considered as marker for peritoneal adhesions and TNF-α antagonists are known to reduce intra-abdominal adhesions [19-20]. TNF-α levels were found to be high in the serum from disease control group. Achnil, but not diclofenac, was able to reduce the level of TNF-α significantly as compared to controls. This suggested that Achnil was able to mitigate the effects of IL-17 in inducing the expression of adhesion molecules and further might also be able to antagonise the effects of TNF-α to a certain extent.

3.5.2 Tumor necrosis factor-α (TNF-α) levels

Significant decline in the levels of TNF-α was observed in the Achnil treated group (G2) as compared to control group on day-14. Treatment with diclofenac upto 14 days was not found to show significant decrease in TNF-α levels as compared to controls (Table 10).

Table 10: Tumor necrosis factor-α levels from all groups

Groups→	**Control**	**Achnil**	**Diclofenac**
TNF-α Levels	**G1**	**G2**	**G3**
	37.4 ± 10.5	20.1 ± 4.0*	30.11 ± 3.7
*Data is presented as mean ±SEM. Unit: IU/ml of serum. * indicates P<0.05 as compared to control group*			

3.6 Gross necropsy and adhesion scoring

The main purpose of subjecting animals to gross necropsy was to identify the presence of neo-adhesions. Observations showed that adhesions were present in almost all animals of the study. However the the percentage area covered by adhesions (APS) was significantly higher in the disease control group.

3.6.1 Adhesion Percentage Scale

Adhesion percentage is shown in Table 11. The results indicated that higher percentage of the abraded tissue exhibited adhesion in the control group. Achnil was able to significantly reduce this adhesion while diclofenac exhibited only modest efficacy in the management of adhesions. Control group (G1) showed severe adhesion up to 75% whereas adhesion percentage was found to be lower in the treated groups.

Table 11: Adhesion Percentage Scale (APS)

Groups→	**Control**	**Achnil**	**Diclofenac**
Adhesion Percentage Scale	**G1**	**G2**	**G3**
	3	1	2
Data is presented as mean values of receptive animals			

It was observed that APS scores from the Achnil treated groups were lower than that of diclofenac and disease control groups. This suggested that the values of serum markers were in close association with gross observations.

The results were further reinforced by the fact that tenacity of the adhesion tissue (indicated by ATTS score) were also significantly higher in the disease control group requiring sharp dissections. Such was also the case with the diclofenac group, however, ATTS scores were found to be lower as compared to controls.

3.6.2 Adhesion Tisse Tenacity Scale

Adhesion Tissue Tenacity Scale is given in Table 12. Dense severe degree of adhesion was observed in control group. Patchy adhesions required equal blunt and sharp dissection. Diclofenac group showed moderate adhesion where as Achnil group showed easily separable adhesion as compared to other groups.

Table 12: Adhesive Tissue Tenacity Scale (ATTS)*

Groups→	**Control**	**Achnil**	**Diclofenac**
Adhesive Tissue Tenacity Scale	**G1**	**G2**	**G3**
	3	0.5	1.5
**Also termed as Degree of Adhesion. Data is presented as mean values of receptive animals*			

In the Achnil treated groups, it was observed that adhesion tissue was not attached firmly to the parietal peritoneum and in most cases the dissection procedure itself led to separation of the tissues from the abdominal walls, whereas in some animals only blunt dissection was required. The gross necropsy findings are shown in the representative images below (Figure 2). It was observed that animals from the control group (G1) showed high degree of adhesion. Animals from the Achnil treated group (G2) showed only mild adhesion whereas those from the diclofenac treated group (G3) showed severe adhesions comparable to control group.

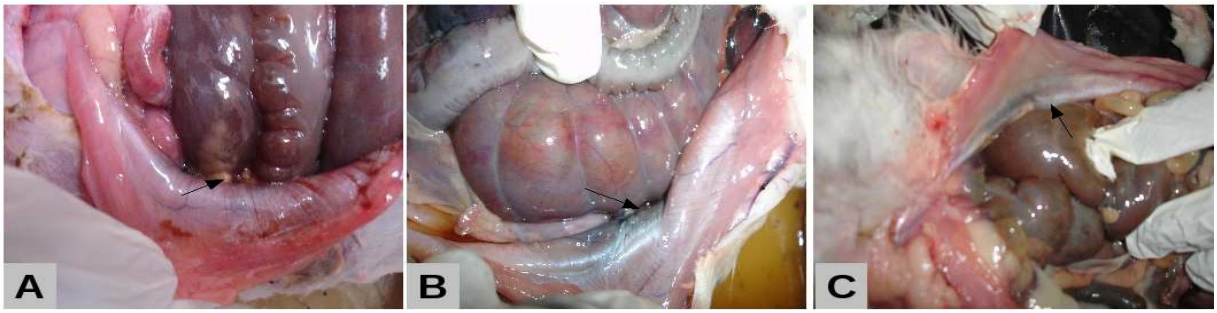

Figure 2: The representative images after necropsy showing the occurrence of adhesion are shown here. A, Control group (G1); B, Achnil treated group (G2); C, Diclofenac treated group (G3). Arrows in A & C indicate the representative adhesion sites observed upon dissection, the arrow in B denotes absence of adhesion at expected site.

3.7 HISTOPATHOLOGY

The concept of inflammatory process during adhesion was also evaluated histopathologically. As shown in microscopic evaluations by Milligan and Raftery (1974), inflammatory infiltration is present in case of adhesions [21]. The H&E stained cells revealed that inflammatory infiltration was severe in the control group on Day-14. Percolation of polymorphonuclear and mononuclear cells was very high in the disease control group. The treated groups were able to reduce the inflammatory infiltrates to a certain extent (Figure 3). Diclofenac exhibited modest potency in this regard whereas Achnil was found to be superior to diclofenac in preventing inflammation. While infiltration was reduced to a certain extent in the diclofenac treated group on Day-14. Achnil treated group showed reduction of inflammatory cell insurgence and a further reduction in inflammatory cell insinuation was recorded microscopically on Day-14 suggesting that Achnil and diclofenac are comparable in restraining the inflammatory process. Achnil group showed that modest inflammation was present which was comparable to the diclofenac group yet lower than the corresponding control group. The effect of surgical induction and respective treatments were graded as a function of inflammatory cell density and quantification. Data is presented in Table 13.

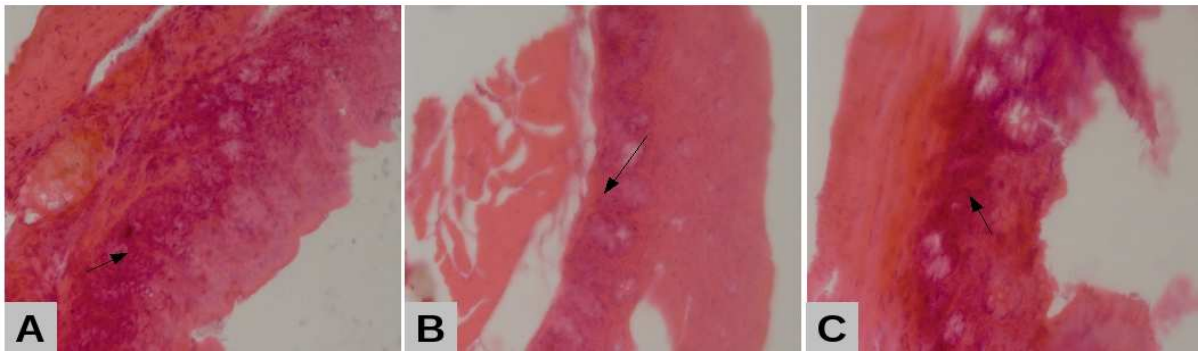

Figure 3: Representative microscopic images of adhesion tissue. All images are stained with H&E (X 400). A, G1-Control group; B, G2-Achnil treated group; C, G3-Diclofenac treated group. Arrows indicate the incidence of infiltrations, which is comparatively mild in the Achnil treated group.

Table 13: Inflammatory cell density and quantitation (by H&E method)

Groups→	**Control**	**Achnil**	**Diclofenac**
Inflammatory cell score	**G1**	**G2**	**G3**
	4	2	2
Inflammatory infiltration was judged by observing the H&E stained slides in a high power field (hpf, X1000). Total number of polymorphonuclear and mononuclear cells were counted per field. Data is presented here as a mean of atleast 3 fields. 0, No inflammatory cells; 1, mild inflammation (4-10 cells/hpf); 2, moderate inflammation (11-50 cells/hpf); 3, severe inflammation (>50 cells/hpf).			

Further degree of adhesion was judged through evaluation of collagen deposition, a precursor to development of inflammatory fibrosis. Formation of the adhesion tissue involves accumulation of collagen within the adhesion tissue [22-23] and this was studied by staining the harvested tissue with Masson's Trichome protocol. Normally, collagen fibres take up greenish-violet color while the cytoplasm and nuclei take red-pink color and the fibrils of elastin take up pink color. Representative images from all the groups are shown in Figure 4.

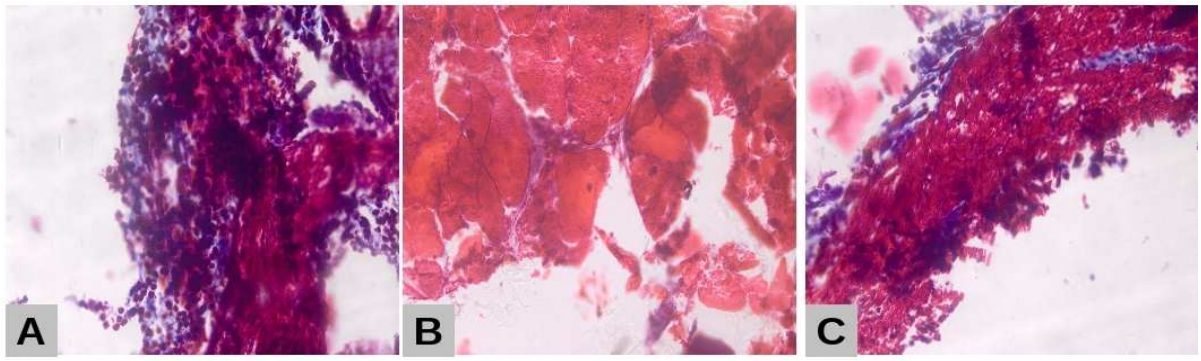

Figure 4: Representative microscopic images of adhesion tissue stained for collagen. All images are stained with Masson's Trichome (X 400). A, G1-Control group; B, G2-Achnil treated group; C, G3-Diclofenac treated group

Microscopic examination revealed that animals from control group showed severe intensity of purple-green coloration. The superior bluish coloration of microscopic fields suggested that the inflammatory process leads to increased formation of adhesion tissue in the disease control groups. Diclofenac group showed less greenish-blue coloration, as compared to control group. Conglomeration of collagen fibers was rather moderate in the diclofenac treated group as compared to the intense accumulation in the control groups. Achnil group showed mild intensity of coloration in some samples. Achnil treated groups amassed minimal collagen fibers which was ultimately an outcome of decreased formation of adhesion tissue.

Overall, the results suggested that Achnil reduces formation of peritoneal adhesions and also reduces the inflammatory cytokines responsible for induction of cellular adhesions. Matrix metalloproteinase and proteoglycan production are key to formation of neo-adhesion tissue at surgical sites. Aceclofenac (the active ingredient of

Achnil), is responsible for blocking interleukins and antagonising the effects of TNF-α. This prevents the production of matrix metalloproteinase and proteoglycan ultimately which may be responsible for prevention of adhesions. It has also been shown that aceclofenac can inhibit adhesion of human neutrophils [17-20].

CONCLUSION

The present study showed that despite once-daily dosing, Achnil offered better protection against development of adhesions. This can be attributed to the prolonged duration of action of Achnil. Overall, Achnil reduced the inflammatory response which may be considered to be a combined effect of COX-inhibition along with blockade of cytokines. Based on the results of the present study, it was concluded that Achnil reduced the formation of post-surgical adhesions in rabbits.

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