

# Histological Effect of Methotrexate and Folinic Acid on Oral Epithelium of Albino Rats

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## ABSTRACT

**Objective:** To determine the extent of damage to oral epithelium caused by high dose methotrexate (HDMTX) and the ameliorating role of folinic acid (FA) on damaging effect of methotrexate.

**Study Design:** Experimental study.

**Place and Duration of Study:** Anatomy Department of Post Graduate Medical Institute (PGMI), Lahore, from March to September 2013.

**Methodology:** Forty-two albino rats were randomly allocated to 3 groups: Exp group-I was given HDMTX intramuscularly (I/M) on alternate days, Exp group-II was given both MTX and FA (I/M) on alternate days, and the control group received no intervention. After 16 days, buccal mucosa was excised for histological analysis under light microscope using H & E stains to see the effect of intervention.

**Results:** Exp group-I showed marked reduction in epithelial thickness compared to Exp group-II, and the control ( $F = 46.44$ ,  $p < 0.001$ ) had significantly depleted basal layer ( $F = 6.32$ ,  $p < 0.004$ ), as well as inflammatory infiltrate with evidence of erosion and ulceration. Exp group-II showed less atrophic changes, a few inflammatory cells, no erosion and ulceration compared to the Exp group-I. Assuming epithelial thickness of control group 100% intact, the Exp group-II was found to have 78% intact and Exp group-I had only 38% thickness intact. Thus FA rescued epithelial thickness by 40%.

**Conclusion:** Folinic acid considerably saved oral mucosa from the damaging effect of HDMTX, improving quality of life of patients.

**Key Words:** Folinic acid. Methotrexate. Oral epithelium. Oral mucositis.

## INTRODUCTION

Oral mucosa maintains its structural integrity by rapid cell division.<sup>1</sup> Chemotherapeutic agents and radiation limit proliferation of epithelium.<sup>2</sup> Methotrexate (MTX), an anti-folate used in high dose in cancer chemotherapy,<sup>3</sup> targets cells that have high mitotic potential, be it malignant cells or healthy oral mucosa cells.<sup>4</sup> Moreover, it inhibits dihydrofolate reductase (DHFR) enzyme thus limits nucleotide synthesis.<sup>5</sup> As a result, purines and pyrimidines are not formed, inhibiting DNA synthesis, which is essential for cell cycle.<sup>6</sup> Its primary toxic effect on rapidly dividing cells poses a significant challenge for oral mucosa.<sup>4</sup> Oral mucositis (OM) is a side effect of MTX which limits one's ability to eat, drink, talk, swallow, and sleep,<sup>4,7</sup> causing direct damage to epithelial cells as well as inflammation and ulceration.<sup>8</sup>

Despite recent advances, cancer therapy-induced mucositis remains a major problem.<sup>9</sup> Anti-inflammatory drugs, corticosteroids, laser therapy, anti-microbial lozenges, ice chips, sucralfate, anti-oxidants and sialogogues have been mentioned as preventive agents

in various studies. However, none has gained large scale consensus among researchers.<sup>10,11</sup>

Folinic acid (FA) is a reduced form of folic acid which does not require reduction by DHFR. FA participates in DNA and RNA synthesis without need of DHFR. It also displaces MTX from DHFR creating supply for fully reduced intracellular folate.<sup>12</sup> It can, therefore, be used as a rescue therapy to counteract methotrexate induced mucositis in animals and humans.<sup>13</sup> A recent update suggested that cryotherapy and keratinocyte growth factor (KGF) prevent oral mucositis.<sup>14</sup> Palifermin, anti-mucotoxic agents, velafermin, probiotics, and IL-11 have been found to reduce oral mucositis in animal models.<sup>15</sup> Sugita *et al.* reported that by taking FA, the incidence was reduced by 25% and severity of the oral mucositis by 28%.<sup>15</sup>

It is claimed that adverse effects of the methotrexate would be reduced by FA considerably, improving quality of life of the subjects consequently.

In view of the above, the present study aimed at assessing the extent of epithelial damage caused by HDMTX and its rescue by FA through histological evaluation of epithelium in albino rats.

## METHODOLOGY

An experimental design used in this study was conducted in PGMI, Lahore, from March to September 2013. Forty-two albino rats were allocated to 3 groups in a

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systematic-random manner such that the first rat was picked up and placed in the control group, second in Exp group-I, and third in Exp group-II. This procedure was continued until all the rats were distributed in three groups. Thus each group got 14 rats. Inclusion criteria comprised of rat weighing 230 - 250 grams and having no oral mucosal lesion by clinical examination. Exclusion criterion comprised of rats whose weight exceeded the weight range of 230 - 250 grams and / or which were clinically found to have any pre-existing oral mucosal lesion.

On day one, MTX 1.56 mg/kg of the body weight of the animal, i.e. 2.4 mg / 250 gms, was injected to Exp groups-I and II.<sup>17</sup> On day two, folic acid 0.1 mg/kg, i.e. 0.15 mg/250 gms, was injected to Exp group-II only.<sup>17</sup> On day three, methotrexate was given to both Exp groups-I and II again. On day four, folic acid was given to Exp group-II only. Same procedure continued till 16th day. Route of administration of both agents was intramuscular. No drug was injected to the control group.

On day 17, rats were anesthetized with ketamine hydrochloride and xylazine and decapitated. Buccal mucosa was dissected by sharp scalpel which was preserved in formalin. The tissues were dehydrated with ethyl alcohol and cleared with xylene. 4µm cut sections of wax blocks were mounted on slides. Histological effects of MTX and FA on buccal tissue of albino rats stained with Haemotoxylin and Eosin (H & E) were seen under light microscope using oculomicrometer. Epithelial and basal layer thickness (µm) was noted by selecting 3 different areas and their mean value was calculated. Then the ratio of basal layer thickness/ epithelial thickness was determined. Inflammatory cells were counted from 3 areas of the slide under same magnification to find the mean value categorized as mild (1), moderate (2), and severe (3). Erosion and ulceration were examined qualitatively and noted as present or absent.

Statistical analysis was carried out with SPSS version 19. Mean and standard deviation values were determined for all the quantitative variables. Qualitative variables were analyzed using chi-square test. Shapiro-Wilk normality test was applied and distribution on all the variables was found to be normal except on inflammation. Kruskal Wallis test was applied where qualitative variable did not meet the condition of normality of data. By dividing the mean values of the control group by the same of Exp groups, multiplied with

100, the effect of drug intervention was worked out in percentage. One-Way ANOVA was applied to find effect of drugs, intervention on the 3 groups followed by least squared difference (LSD) post-hoc analysis. F-test statistic was applied for the cutoff significance ( $p < 0.05$ ).

## RESULTS

F-statistic revealed systematic and significant difference ( $p < 0.01$ ) among Exp groups-I and II, and the control group. Mean and standard deviation of 3 groups/ conditions is described in Table I to showcase intervention effects.

Epithelial thickness of the control group had the highest mean thickness value and it also showed well layered keratinocytes. Exp group-I, which took MTX only, showed most reduced or damaged epithelial thickness. Keratinocytes also showed atrophy of epithelium as size was reduced. Exp group-II, which took MTX as well as folic acid, possessed comparatively far more epithelial thickness than Exp group-I, and had well layered keratinocytes with proper morphology. FA significantly rescued the buccal mucosa of rats from the dwindling effects of MTX (66.96 µm vs. 31.87 µm) and saved epithelial thickness considerably.

Comparatively low variation in epithelial thickness of control group was due to the fact that they had not been subjected to drugs, intervention. It also showed highest basal layer thickness and cuboidal to columnar shaped cells compared to Exp group-I whose basal layer was significantly reduced and it was lacking in distinct cuboidal shaped cells for taking HDMTX (Table I). The status of the Exp group-II was in-between the other 2 groups for taking MTX with FA. In fact, it showed intact basal layer close to the mean index of the control group. As mitotic cells were also seen here, it indicated that the repair process had restored the basal layer.

The ratio of basal layer thickness to epithelial thickness was found to be highest in Exp group-I, due to reduction in epithelial thickness with the use of MTX. However, Exp group-II, treated with both MTX and FA simultaneously, attained a mean thickness value close to that of the control group. The maximum difference on all the 3 variables, namely epithelial thickness, basal thickness, and ratio of basal to epithelial thickness, were found between Exp group-1 and II contrast groups as different treatment conditions had been meted out to them (Table III).

**Table I:** One way ANOVA: Difference between groups on study variables (N=42).

Variables	Exp Group-I (n=14)	Exp Group-II (n=14)	Control Group (n=14)	F	p-value
Epithelial thickness (µm)	31.87±15.92	66.96±19.84	85.50±4.83	46.44	0.001
Basal thickness (µm)	5.37±2.54	7.27±2.26	8.00±0.28	6.32	0.004
Ratio: Basal thickness/ Epithelial thickness	0.17±0.05	0.10±0.30	0.09±0.008	20.59	0.001

F = F-statistic in the above ANOVA displays significance of difference in the scores of the 3 groups.

**Table II:** Effects of different drug conditions/groups on study variables: A comparison.

Variables	Conditions		Effect*
Epithelial thickness	Control	---	100% intact
	Control	Exp-I	37% intact
	Control	Exp-II	78% intact
Basal layer thickness	Control	---	100% intact
	Control	Exp-I	67% intact
	Control	Exp-II	90% intact

\* Intervention effect in % = Mean of experimental group / Mean of control group X 100

**Table III:** LSD comparison on mean difference between any pair of groups.

Variables	Groups	Mean Difference	Significance/ p-value
Epithelial layer			
Exp-I	Exp-II	-35.083	0.001
Exp-I	Control	-53.001	0.001
Exp-II	Control	-18.537	0.014
Basal layer			
Exp-I	Exp-II	-1.870	0.019
Exp-I	Control	-2.646	0.001
Exp-II	Control	-0.776	0.316
Basal / Epithelial			
Exp-I	Exp-II	0.069	0.001
Exp-I	Control	0.085	0.001
Exp-II	Control	0.016	0.251

LSD = Least square difference, N = 14 subjects in each group.

Inflammation was highest in Exp group-I with mean rank of 34.43 followed by Exp group-II where it was moderate (18.36). The control group exhibited still milder inflammation with mean rank 11.71 and very few inflammatory cells, mainly lymphocytes were seen. Kruskal Wallis test showed difference in inflammation of the 3 groups as significant  $\chi^2 (2) = 29.02, p < 0.001$ . These results support the hypotheses that methotrexate caused inflammation in group-I, and folic acid rescued it in group-II.

A chi-square test of independence revealed significant relation between drug intervention and erosion of mucosa among the 3 groups  $\chi^2 (3, N = 42) = 19.76, p < 0.001$ . Eight animals (57%) in Exp group-I (given MTX only) were found to have erosion in their epithelium, whereas none of the animals in Exp group-II suffered from erosion in epithelium (given MTX and FA). Similar analysis for ulceration was found not significant  $\chi^2 (3, N = 42) = 8.84, p < .12$ . Rats of Exp group-I showed ulceration as entire thickness of epithelium was lost. However, none of the animals in Exp group-II showed any signs of ulceration.

Table II shows that epithelial layer of Exp group-I, which took MTX only, remained only 37% intact whereas that of the Exp group-II was 78%. The latter was consequently rescued by 41%, with the use of FA. The basal layer of Exp group-I was 67% in thickness of what it was in the control group, i.e. 33% worse in the former. Comparatively, Exp group-II was better off in saving basal layer to the extent of 90% of what it was in the

control group. So the extent of rescue accounted for by folic acid thus coming to 23% (67% - 90%) testifying the efficacy of folic acid as a rescuing agent.

## DISCUSSION

Oral mucositis is a common side effect of methotrexate therapy.<sup>7</sup> When MTX is used in high dose, it has a substantial clinical impact on patient's quality of life.<sup>16</sup> Alternately, dose modification or discontinuation of the treatment, would likely cause an extended stay in hospital.<sup>10</sup>

Rat's keratinized stratified squamous oral epithelium, with highest mean epithelial thickness and columnar shaped basal cells of control group, served as a standard condition to compare mucosae of experimental animals under intervention conditions. The evidence found in this experiment has supported the use of folic acid for protecting oral epithelium from damaging effects of high dose MTX therapy. Rescue by folic acid is attributed to its mechanism of action that it reduces the mucosal damage and promotes proliferation of epithelial cells without affecting the medicine's activity.<sup>13</sup> Sultan *et al.* also found epithelial thickness in controls as much higher compared to that of the experimental subjects.<sup>18</sup>

Stratified squamous epithelium of Exp group-I rats, who took MTX, showed atrophy along with flattening or shortening of rete ridge, something not visible in control group since the epithelial thickness was much higher there along with intact multilayered keratinocytes. Decreased epithelial thickness is attributed to reduction in proliferative potential of epithelium by methotrexate. Munaretto *et al.* reported similar findings where mice were immune suppressed with the sub-cutaneous injections of 2.5 mg/kg MTX for 3 consecutive days. The epithelial thickness of the tongue mucosa decreased as duration of study increased, however, inflammation and ulceration were not found in their study.<sup>19</sup>

Experimental animals of the present study showed moderate to severe inflammation with predominant macrophages along with neutrophils and lymphocytes. Ulcers were also seen in few animals as a corroborating support for damaged oral epithelium. Jenson *et al.* made similar findings upon clinical observation and reported that oral mucositis was present in 44% of the patients as a sequel of chemotherapy.<sup>20</sup>

Reduction in the basal layer thickness is attributed to death of basal cells in Exp group-I which have replicative potential. Colonogenic death of basal epithelial stem cells leads to reduction in renewal capacity of epithelium under high dose of methotrexate causing ulceration.<sup>8</sup>

The present study, being *in vivo*, reported the viability of epithelium and basal layer with the use of FA as 77% and 90%, respectively in Exp group-II, assuming that of the control group as 100% intact. This study draws

support from Maiguma who conducted an *in vitro* study on the epidermal keratinocytes and periodontal ligament fibroblasts. It reported that MTX caused damage only to keratinocytes that were in epithelium and not to fibroblasts which reside in connective tissue. Epidermal cells were found intact up to 78% with the use of folic acid which had been reduced to 56% in the other group taking MTX alone.<sup>21</sup>

Similar to this study, Sugita *et al.* found that oral mucositis caused by HDMTX in a span of 6 days was rescued with FA by 24% along with other supportive care namely G-CSF, levofloxacin, and anti-viral agents.<sup>16</sup> The present study is, however, different in several ways. First, MTX was given intramuscularly over a long course of 15 days. Secondly, it involved healthy / disease-free albino rats that had no previous history / disease effects. Thirdly, patients in the Sugita's study had undergone hematopoietic stem cell transplant and were immunocompromised. Fourthly, prevention of oral mucositis was carried out by FA only (without any supportive therapy). Despite these variations, this study can be considered as a replicative study that established rescue of the epithelial thickness by 41% and that of basal layer by 23% with the use of folic acid.

A meta-analysis by Worthington *et al.* showed significant beneficial evidence for prevention or reduction of oral mucositis in cancer treatment from cryotherapy, keratinocyte growth factor and sucralfate.<sup>14</sup> Interestingly, these did not include folic acid as an agent fighting out for mucositis. The present study provides strong evidence for the utility of FA as a rescuing agent in preventing oral mucositis.

## CONCLUSION

The present study supported the twin hypotheses. Folic acid not only maintained epithelium intact which otherwise would have diminished due to methotrexate. but also reduced inflammation. The rescuing effect of folic acid was impressive as the epithelial thickness remained intact to the extent of 78%. This can tremendously enhance the quality of life among cancer patients who have to take MTX in high doses.

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