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Exploration of a novel adjuvant therapeutic regimen using a potent glucocorticoid receptor agonist along with iNOS inhibitor in murine model of asthma

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ABSTRACT

Allergic asthma is mainly characterized by allergen-induced IAR (immediate airway response) and LAR (late airway response). In regards to the results of lung tissue histology and bronchoalveolar lavage fluid analysis, it was confirmed that there is the existence of a casual relationship of eosinophil and other inflammatory cell infiltration in bronchial sub-mucosa in the mechanism of LAR. This investigation aimed to examine the anti-asthmatic effect of novel adjuvant therapeutic regimens using a low dose of potent glucocorticoid receptor agonist i.e., Dexamethasone, along with iNOS inhibitor i.e., Aminoguanidine in a both acute and chronic murine model of asthma. Female BALB/c mice of 8 weeks of age were taken and divided into 6 experimental groups i.e. normal control, OVA control, aminoguanidine (200mg/kg), combination of aminoguanidine (200mg/kg) along with dexamethasone (0.03mg/kg), low dose dexamethasone (0.03mg/kg) and Dexamethasone (0.1mg/kg) treated group. After sensitization and introduction of drugs, mice were sacrificed by cervical dislocation and bronchoalveolar lavage (BAL) fluid analyzed. The result of this study stated that there is a significant reduction in the levels of inflammatory cytokines in combination-treated animals with respect to alone dexamethasone, which may be due to the synergistic effect of aminoguanidine and dexamethasone. From histopathological evidence, it can be concluded that combination treatment having a better lung adaptation mechanism and can improve the condition aggressively. The findings of the study throw some light on an additive therapeutic regimen of aminoguanidine can have a better impact with glucocorticoids.

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INTRODUCTION

Allergic asthma is mainly characterized by allergen-induced immediate airway response (IAR) and late airway response (LAR). It remains a complicated disorder of the airways resultant of inflammation. A hallmark feature of asthma is the presence and activation of inflammatory cells in the airways, notably eosinophils, basophils, mast cells, and T lymphocytes, and stimulation of structural/resident cells, including those of the airway epithelium, fibroblasts, smooth muscle, goblet cell hyperplasia, fibroblasts and TGF-β1 plays an important role in such condition (Duvernelle *et al.*, 2003). Histopatho-

logical evaluation and analysis of bronchoalveolar lavage fluid (BALF) of lung tissue revealed that the infiltration of eosinophils and other inflammatory cells into the bronchial submucosa mainly responsible for LAR (Monchy *et al.*, 1985). Inflammatory mediators like pro-inflammatory cytokines, nitric oxide (NO) and cysteinyl leukotrienes (Cys LTs) involved in a decisive role of establishing LAR (Meurs *et al.*, 2003). Glucocorticoids, along with B2 agonist are widely used to treat various inflammatory lung diseases (Simons, 1997; Jonasson, 2000). Glucocorticoids exert its mechanism through the glucocorticoid receptor (GR) by inflecting either repress (transrepression) or induce (transactivate) gene transcription. In asthma, there is needed long term therapy of inhaled glucocorticoids for its local anti-inflammatory effect, but which can goes to the systemic circulation through the lungs and produces its side effects (Wolthers and Allen, 2002; Randell *et al.*, 2003).

Like all other asthmatic mediators, it has been reported that in asthma high amount of nitric oxide (NO) is produced by inducible nitric oxide synthase (iNOS) induced by bacterial products and cytokines, and this NO acts as a regulatory and pro-inflammatory mediator (Agard *et al.*, 2009). Elevated Nitric oxide (NO) levels are evidence of up-regulated iNOS expression in the airways of asthmatics. The use of a selective iNOS inhibitor significantly reduces NO exhaled by asthmatics (Crater *et al.*, 1999). It is reported that iNOS-deficient mice shown less prone to Allergic inflammation in the lungs (Xiong *et al.*, 1999). NO has distinct effects on the immune system and effectively modulates inflammatory responses. It suppresses T cell proliferation and Th1 cytokine production in mice and thus favors the development of Th2 response with eosinophilia and proceeding to lgE production and promotion can cause asthma (Barnes and Liew, 1995). High concentration NO combined with superoxide (0+2) to produce the highly toxic peroxynitrite anions (OONO-) and hydroxyl radicals, which produce high oxidative stress and may damage airways epithelium and promote inflammation (Coleman, 2001). For these reasons, it is likely that the selective inhibition of iNOS in asthma will result in decreased pulmonary inflammation and improved airway function. Increasing evidence in various animal models of asthma with either selective iNOS inhibitors or iNOS gene disruption supports this concept (Hansel and Barnes, 2001). It may also promote glucocorticoids receptor bounding action, which will be a new pharmacological strategy for improving the efficacy and therapeutic ratio of glucocorticoids in inflammatory lung diseases like chronic asthma. By cumulating all these above information, we select a specific INOS inhibitor (Aminoguanidine PO), which may produce an anti-asthmatic effect itself and may also contribute "Add-on Therapy" along with a low dosage of selective GR agonist (Dexamethasone, PO) in an ovalbumin-induced murine model of chronic asthma.

MATERIALS AND METHODS

Animals used

Female BALB/c mice of approximately 8 weeks aged were selected for this study. Animals were kept in the standard environmental condition specified by CPCSEA. Appropriate food and drinks were supplied to animals. Institutional Animal Ethics Committee (IAEC) reviewed the protocol and approved conducting this study.

STUDY DESIGN

The animals were acclimatized to experimental room conditions for 2 days before initiation of the study. They were divided into six groups (n=6) as per below.

Group-1- Normal control

Group-2- OVA control

Group-3- iNOS inhibitor (Aminoguanidine 200mg/kg, PO, Two times per day)

Group-4- iNOS inhibitor + GR agonist (Aminoguanidine 200mg/kg, PO, Two times per day + Dexamethasone 10mg/kg)

Group-5- Low dose GR agonist (Dexamethasone 0.03mg/kg)

Group-6- High dose GR agonist (Dexamethasone 0.1mg/kg)

Experimental

Statistical analysis

Values for biochemical parameters were expressed as mean $\pm$ S.E.M. Statistical significance was calculated using one way ANOVA followed Dunnett's test.

RESULTS AND DISCUSSION

The WBC count in the normal animal was 0.262 ± 0.5x$103/\mu$l while after OVA challenge it went up to 1.318 ± 0.36 x$103/\mu$l. Oral administration of aminoguanidine, aminoguanidine with low dose of Dexamethasone (0.03mg/kg), Low dose of Dexamethasone (0.03mg/kg), and Dexamethasone (0.1mg/kg) treated showed WBC

Table 1: Effect of different drugs on WBC count

Groups	Treatments	Dose(p.o)	WBC Count($\times 10^3$ /μl)
1	Normal Control	NA	0.262±0.5
2	OVA Control	NA	1.318±0.36
3	Aminoguanidine	200mg/kg(BID)	0.732±0.09
4	Aminoguanidine + Dexamethasone	200mg/kg(BID)+ 10mg/kg	0.285±0.04
5	Dexamethasone	0.03mg/kg	0.399±0.036
6	Dexamethasone	0.1mg/kg	0.399±0.085

Table 2: Effect of different drugs on Eosinophils count

Groups	Treatments	Dose(p.o)	Eosinophils count ($\times 10^3$ /μl)
1	Normal Control	NA	0.118±0.00
2	OVA Control	NA	0.436±0.01
3	Aminoguanidine	200mg/kg(BID)	0.350±0.09
4	Aminoguanidine + Dexamethasone	200mg/kg(BID)+ 10mg/kg	0.057±0.00
5	Dexamethasone	0.03mg/kg	0.560±0.02
6	Dexamethasone	0.1mg/kg	0.526±0.03

Table 3: Effect of different drugs on Monocytes count

Groups	Treatments	Dose(p.o)	Monocytes count ($\times 10^3$ /μl)
1	Normal Control	NA	0.032±0.01
2	OVA Control	NA	0.305±0.14
3	Aminoguanidine	200mg/kg(BID)	0.113±0.01
4	Aminoguanidine+ Dexamethasone	200mg/kg(BID)+10mg/kg	0.049±0.011
5	Dexamethasone	0.03mg/kg	0.044±0.036
6	Dexamethasone	0.1mg/kg	0.020±0.01

Table 4: Effect of different drugs on Lymphocyte count

Groups	Treatments	Dose(p.o)	Lymphocyte count ($\times 10^3$ /μl)
1	Normal Control	NA	0.145±0.04
2	OVA Control	NA	0.611±0.13
3	Aminoguanidine	200mg/kg(BID)	0.444±0.08
4	Aminoguanidine+ Dexamethasone	200mg/kg(BID)+10mg/kg	0.148±0.04
5	Dexamethasone	0.03mg/kg	0.235±0.02
6	Dexamethasone	0.1mg/kg	0.181±0.04

Table 5: Effect of different drugs on Neutrophils count

Groups	Treatments	Dose(p.o)	Neutrophils count ($\times 10^3$ /μl)
1	Normal Control	NA	0.042±0.04
2	OVA Control	NA	0.223±0.13
3	Aminoguanidine	200mg/kg(BID)	0.126±0.02
4	Aminoguanidine+ Dexamethasone	200mg/kg(BID)+10mg/kg	0.048±0.01
5	Dexamethasone	0.03mg/kg	0.083±0.02
6	Dexamethasone	0.1mg/kg	0.134±0.05

Table 6: Effect of drugs on tumor necrosis factor-α inBAL fluid

Groups	Treatments	Dose(p.o)	TNF-α (pg/ml)
1	Normal Control	NA	301.31±34.34
2	OVA Control	NA	438.46±33.85
3	Aminoguanidine	200mg/kg(BID)	279.43±71.57
4	Aminoguanidine+ Dexamethasone	200mg/kg(BID)+10mg/kg	369.08±41.92
5	Dexamethasone	0.03mg/kg	279.43±71.56
6	Dexamethasone	0.1mg/kg	378.06±1.83

Table 7: Effect of drugs on Interleukin-6 (IL-6) in BAL fluid

Groups	Treatments	Dose(p.o)	IL-6 ($\times 10^3$ /μl)
1	Normal Control	NA	70.08±50.06
2	OVA Control	NA	103.51±18.33
3	Aminoguanidine	200mg/kg(BID)	62.43±4.67
4	Aminoguanidine+ Dexamethasone	200mg/kg(BID)+10mg/kg	48.71±23.03
5	Dexamethasone	0.03mg/kg	56.76±19.09
6	Dexamethasone	0.1mg/kg	129.09±14.39

Table 8: Effect of drugs on Interferon-γ (IFN-γ) in BALfluid

Groups	Treatments	Dose(p.o)	IFN-γ ($\times 10^3$ /μl)
1	Normal Control	NA	305.55±22.5
2	OVA Control	NA	300.09±70.22
3	Aminoguanidine	200mg/kg(BID)	206.29±21.4
4	Aminoguanidine+ Dexamethasone	200mg/kg(BID)+10mg/kg	107.49±46.3
5	Dexamethasone	0.03mg/kg	249.06±16.29
6	Dexamethasone	0.1mg/kg	261.52±11.95

counts 0.732±0.09, 0.285±0.04, 0.399±0.036, 0.399±0.085 respectively and were shown in Table 1. The eosinophil count in the normal animal was 0.118±0.00x103/μl while after OVA challenge it went up to 0.436± 0.01 x103/μl. Oral administration of aminoguanidine, aminoguanidine with low dose of Dexamethasone (0.03mg/kg), Low dose of Dexamethasone (0.03mg/kg), and Dexamethasone (0.1mg/kg) treated showed eosinophil counts 0.350±0.09, 0.057±0.00, 0.560±0.02, 0.526±0.03 respectively and depicted in Table 2. The monocytes count in the normal animal was 0.032±0.01x103/μl while after OVA challenge it went up to 0.305±0.14 x103/μl. Oral administration of aminoguanidine, aminoguanidine with low dose of Dexamethasone (0.03mg/kg), Low dose of Dexamethasone (0.03mg/kg), and Dexamethasone (0.1mg/kg) treated showed monocyte counts 0.113±0.01, 0.049±0.011, 0.044±0.036, 0.020±0.01 respectively and were shown in Table 3. The lymphocyte count in the normal animal was 0.145±0.04x103/μl while after OVA challenge it

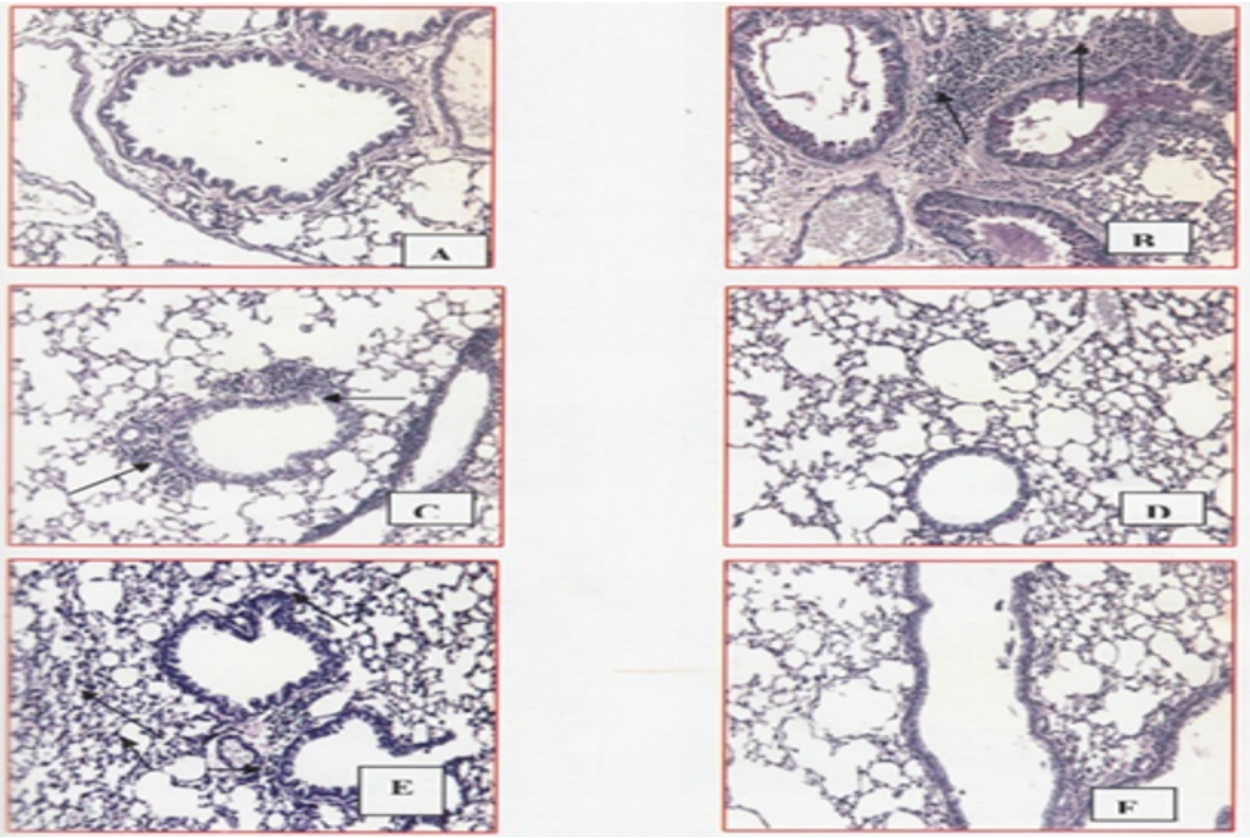

Figure 1: H&E Staining. A - Normal Control. B - Inflammatory Cells. C - Aminoguanidine (LessInflammatory Cell).D - Aminoguanidine+Dexamethasone. E - Dexamethasone 0.03 mg/kg (LessInflammatory Cell).F - Dexamethasone 0.1 mg/kg

went up to 0.611±0.13x103/μl. Oral administration of aminoguanidine, aminoguanidine with low dose of Dexamethasone (0.03mg/kg), Low dose of Dexamethasone (0.03mg/kg), and Dexamethasone (0.1mg/kg) treated showed lymphocyte counts 0.444±0.08, 0.148±0.04, 0.235±0.02, 0.181±0.04 respectively and were shown in Table 4. The neutrophil count in the normal animal was 0.042±0.01x103/μl while after OVA challenge it went up to 0.223±0.05 x103/μl. Oral administration of aminoguanidine, aminoguanidine with low dose of Dexamethasone (0.03mg/kg), Low dose of Dexamethasone (0.03mg/kg), and Dexamethasone (0.1mg/kg) treated showed neutrophil counts 0.126±0.02, 0.048±0.01, 0.083±0.02, 0.134±0.05 respectively and were shown in Table 5.

The TNF-α count in the normal animal was 301.31±34.34 pg/ml, while after the OVA challenge, it went up to 438.46±33.85 pg/ml. Oral administration of aminoguanidine, aminoguanidine with a low dose of Dexamethasone (0.03mg/kg), Low dose of Dexamethasone (0.03mg/kg), and Dexamethasone(0.1mg/kg) treated showed TNF-α counts were 279.4±71.57, 369.08±41.92, 279.43±71.56, 378.06±1.83 pg/ml respectively and represented in Table 6.

The IL-6 count in the normal animal was 70.08±50.06 pg/ml, while after the OVA challenge, it went up to 103.51±18.33 pg/ml. Oral administration ofaminoguanidine, aminoguanidine with a low dose of Dexamethasone (0.03mg/kg), Low dose of Dexamethasone (0.03mg/kg), and Dexamethasone (0.1mg/kg) treated Shows IL-6 counts were 62.43±4.67, 48.71±23.03, 56.76±19.09, 129.09±14.39 pg/ml respectively and represented inTable 7.

The IFN-γ count in the normal animal was 305.55±22.5 pg/ml, while after the OVA challenge, it went up to only 300.09±70.22 pg/ml. Oral administration of aminoguanidine, aminoguanidine with a low dose of Dexamethasone (0.03mg/kg), Low dose of Dexamethasone (0.03mg/kg), and dexamethasone (0.1mg/kg) treated Showed IFN-γ counts were 206.29±21.4, 107.49±46.3, 249.06±16.29, 261.52±11.95 pg/ml respectively and represented in Table 8.

HISTOPATHOLOGICAL EVALUATION

H&E staining

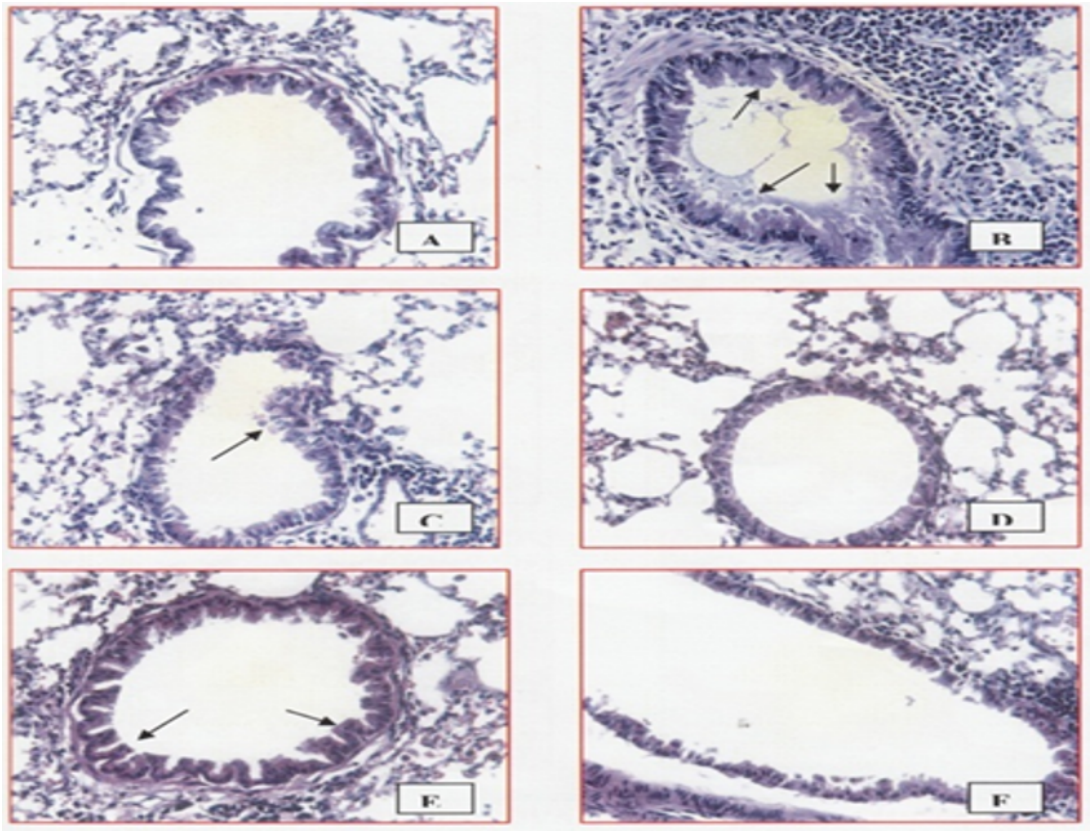

Figure 2: PAS Staining. A- Normal Control. B- Profound Goblet Hyperplasia Cells (PGHC). C - Aminoguanidine (PGHC). D - Aminoguanidine+ Dexamethasone. E - Dexamethasone 0.03 mg/kg (PGHC).F- Dexamethasone 0.1 mg/kg

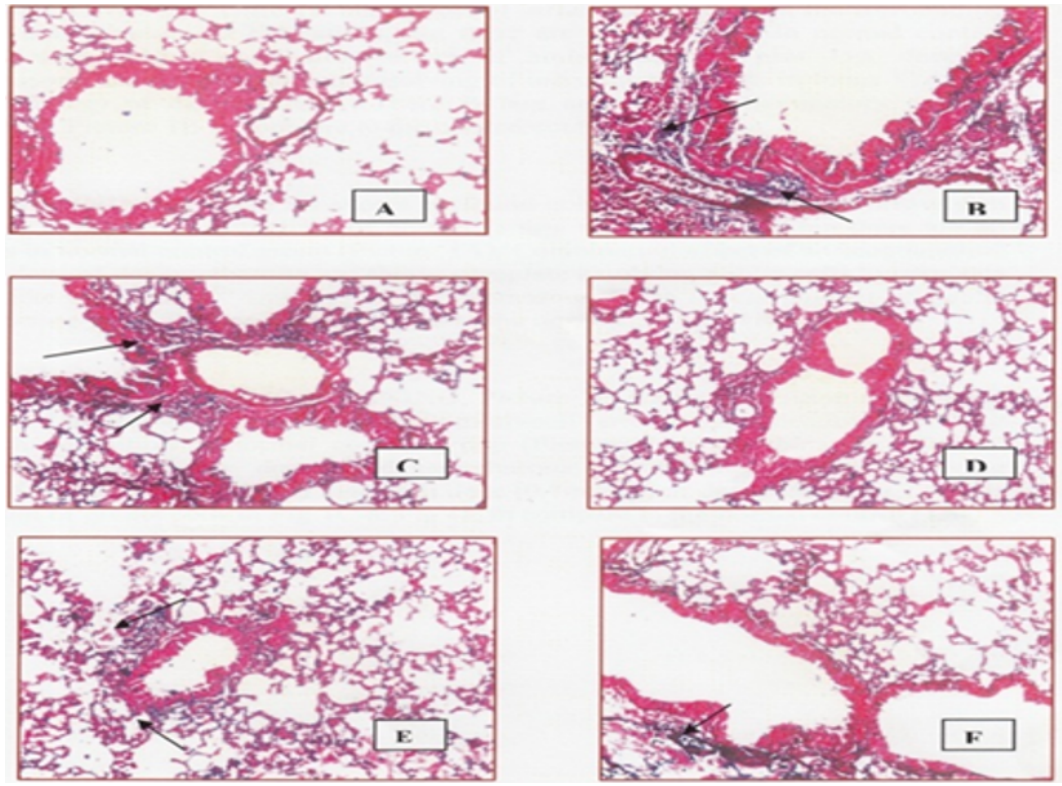

Figure 3: MASSON'S Trichome Staining. A- Normal Control. B- OVA Control. C- Aminoguanidine. D- Aminoguanidine+ Dexamethasone. E- Dexamethasone 0.03 mg/kg. F- Dexamethasone 0.1 mg/kg

Figure 1 shows H&E staining, Figure 1B shows lots of inflammatory cells in the surrounding of the alveoli (arrow sign indication), but there are no such cells in the normal control group (Figure 1A). The combination effect of aminoguanidine plus a low dose of 1 dexamethasone is showing complete resolving of inflammatory cells in the lungs (Figure 1D) like a high dose (0.1mg/kg) of dexamethasone. There are fewer amounts of inflammatory cells in Figure 1C &Figure 1E in compare to normal control (Figure 1A).

PAS staining

Figure 2 shows the PAS staining. Figure 2B shows profound goblet cell hyperplasia (arrow sign indication) in the inner epithelium of alveoli (arrow sign indication). Still, there are no such cells in the normal control group (Figure 2A). The combination effect of aminoguanidine plus a low dose of dexamethasone is showing complete resolving goblet cell hyperplasia in lungs (Figure 2D) like a high dose (0.1mg/kg) of dexamethasone. There are fewer amounts of goblet cells in Figure 2C & Figure 2E in compare to normal control.

Masson's Trichome staining

Figure 3shows Masson's Trichome staining. Figure 3B shows the deposition of collagen (takes blue staining) in the outer epithelium of alveoli (arrow sign indication). Still, there is no such deposition in the normal control group (Figure 3A). The combination effect of aminoguanidine plus a low dose of dexamethasone is shown complete resolving collagen deposition in lungs (Figure 3D) than the high dose (0.1mg/kg) of dexamethasone. There is less amount of goblet cells in Figure 3C & Figure 3E in comparison to the normal control.

In the present study, we investigated the effect of aminoguanidine and dexamethasone in a model of the allergen (OVA) induced asthma in mice. In this study, we have shown that the OVA challenge in mice induces a significant elevation of WBC in the BALF. The Ova challenge resulted in excess production of neutrophils and eosinophils associated with key inflammatory cytokines, including TNF-α, IL-6 and IFN-γ in BALF. Our findings support several earlier studies that have demonstrated the accumulation of eosinophils and neutrophils and an increase of pro-inflammatory cytokines levels in the airways and BALF (Sr, 2007; Kharitonov *et al.*, 1996). The findings of this research revealed that cytokines levels were significantly decreased in combination-treated animals with respect to alone dexamethasone treatments, which may be due to the synergistic effect of aminoguanidine and dexamethasone. The pharmacological mechanism of this synergistic effect may be due to the elevation of HDAC2 activity in combination-treated animals than OVA control animals (Ito *et al.*, 2005). Especially IL-6 level was significantly decreased in combination-treated animals, but interestingly IL-6 level was increased in a high dose of dexamethasone-treated animals. It was observed that both aminoguanidine and dexamethasone as monotherapy significantly decreased total count and eosinophil counts in the BAL fluid. However, no significant change in cell count was observed in the combination treatment group in comparison to single treatments. Histopathological data showed combination treatment completely resolves the infiltrating inflammatory cells into the lungs.

Furthermore, combination treatment could able to reduce goblet cell hyperplasia and collagen deposition in lamina propria of alveoli. Infiltration of inflammatory cells can cause structural changes includes sub-epithelial fibrosis in asthmatic airways. The infiltration of inflammatory cells, goblet cell hyperplasia and collagen deposition, are closely associated with the level of cytokines (Wen *et al.*, 2003; Riffo-Vasquez and Spina, 2002), which was markedly inhibited by this combination therapy than OVA control group.

CONCLUSIONS

By cumulating all the findings of current research, this pre-clinical study concluded that combination treatment of aminoguanidine and dexamethasone showed better recovery of lung inflammation and remodeling in asthmatic animals which is a new pharmacological strategy for improving the efficacy and therapeutic ratio of glucocorticoids in inflammatory lung diseases like chronic asthma. This study may enlighten a novel combination therapy to ameliorate asthma and other inflammatory lung diseases like COPD, which needs further clinical investigation.

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