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Full length Research Paper

Expression of glucocorticoid receptors in Nasal Mucosa of Patients with Allergic Rhinitis under local Intranasal Corticosteroid Therapy

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The expression level of glucocorticoid receptor (GR) isoforms α and β might interfere with the response to exogenous glucocorticoids (GC) in different allergic disorders. No much data concerning allergic rhinitis (AR) currently exists. The aim of this study was to detect the change in the expression level of GRα in the nasal mucosa of AR patients, compared to healthy subjects, following 4 weeks of intranasal GC application, and whether this change was correlated to the clinical response of those patients.Twenty AR patients (mean age 31.3 ± 8.7) and 10 healthy volunteers (mean age 32.5 ± 17.7) were enrolled in this clinical trial. Intranasal mometasone furoate was applied for all participants for 4 weeks. The level of GRα expression, before and after treatment, was compared by quantitative real time PCR. Total nasal symptom score (TNSS) was used to assess the clinical response of AR patients. This study revealed that GRα was down-regulated in 85% of AR patients compared to 100% of healthy volunteers. Mann Whitney-U test (MW) revealed insignificant difference in the mean fold change (FC) of GRα expression between AR patients and healthy subjects (0.8±1.1 compared to 0.4±0.2) (P0.1). Chi squared test revealed decreased GRα expression among higher ratio (92.3%) of patients with residual symptoms after treatment than those with total improvement (71.4%), which was insignificant statistically (P0.2). MW revealed more decrease in GRα expression in patients with residual symptoms (mean FC 0.8 ± 1.3), compared to patients that were totally improved (mean FC 0.9 ± 0.6), that was statistically insignificant (P0.2). The test also revealed a significant (P 0.0) decrease of TNSS of AR patients after treatment, though weakly correlatedto the change in GRα expression with Spearman correlation coefficient of -0.2 (P0.3). Therefore, GRα expression in AR patients, following 4 weeks of intranasal GC, is variable and might not be a contributing factor determining the response of AR patients to exogenous GC.

Key words: Glucocorticoid receptors; Allergic rhinitis; Intranasalglucocorticoids.

INTRODUCTION

Allergic rhinitis (AR) is an inflammatory condition of nasal mucosa characterized by hyper-responsiveness, over-production of type 2 helper (T_H2) cytokines and intense inflammatory cell recruitment, predominantly eosinophils (Kay 2001).

In sensitized individuals, it is elicited by an interaction between environmental allergens and immunoglobulin

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(Ig) E and is characterized by nasal symptoms as congestion, sneezing, rhinorrhea, itching and anosmia as well as ocular manifestations which could result in considerable deterioration of the quality of life (Sapsaprang et al. 2015).

Currently, intranasal glucocorticoids (GC) constitute the single most effective medications available for the management of AR. Furthermore, they have been strongly recommended, as first line treatment, over H1-antihistamines and leukotriene receptor antagonists for treating AR in adults (Brozek et al. 2010). Nevertheless, some concerns have been raised about the occurrence of resistance in some patients (Luo et al. 2007).

GC have a broad anti-inflammatory effect regulating both innate and adaptive immune responses in a wide variety of cells. This is achieved mainly through the regulation of expression of proteins associated with inflammation at both the transcriptional and the post-transcriptional levels (Pelaia et al. 2003; Liberman et al. 2007; Stellato 2007).

Though not completely understood, the GC anti-inflammatory response is mediated through a 94-KDa cytoplasmic protein called glucocorticoid receptor (GR). Alternative splicing of GR gene gives rise to different GR isoforms, with GRα being the most predominant and active one (Liberman et al. 2007). After its binding with GCs, the GR-GCs dimer is able to translocate to the nucleus exerting its function as a transcription factor (Liberman et al. 2007).

GR expression is known to be down-regulated by different GC in different cell types and tissues including nasal mucosa of healthy subjects (Knutsson et al. 1996). In contrast, conflicting results were reported when inflamed nasal mucosa (nasal polyps) was studied. Meanwhile no down-regulation of GR mRNA after steroid treatment was reported by some authors (Henriksson et al. 2001; Pujols et al. 2008), down-regulation was reported by others, particularly, when GRα was studied (Li et al. 2010).

Though the down-regulation of GRα in healthy nasal mucosa is thought to be a protector mechanism that would avoid the deleterious effects of prolonged exposure to GC, it is not surprising that this may decrease cell sensitivity to exogenous GC, which is partly dependent on the number of receptors found in the cell (Gross et al. 2009).

This assumption was extensively studied previously. In most of published studies, it was concluded that the imbalance in GRα expression in relation to other isoforms, particularly GRβ, was found to be correlated to GC resistance in different allergic and non-allergic disorders, including nasal polyps (Sousa et al. 2000; Hägg et al. 2009; Li et al. 2010; Ma et al. 2013).

Concerning AR, no much data is currently available. Yet, a previous study demonstrated that excessive expression of GRβ in peripheral blood mononuclear cells (PBMC) might be a predictor of GC resistance in AR patients (Luo et al. 2007).

For better understanding of the role played by GRα with intranasal GC, this study has been carried out in order to detect the changes in the level of GRα expression in nasal mucosa of AR patients following 4 weeks of intranasal GC application and to detect whether this change, if any, is correlated to the clinical response of those patients.

MATERIALS AND METHODS

This study was conducted in the ENT & Microbiology and Immunology Departments and Zagazig Medical Scientific Research Centre, Faculty of Medicine, Zagazig University, Egypt, in the period between March 2013 and March 2014. Twenty AR patients (12 male and 8 females) were enrolled in this study. Their ages ranged from 18 to 45 years (mean 31.3 ± 8.7). They were selected by systemic random sampling from patients attending the ENT outpatient clinic at Zagazig University Hospitals, Egypt. Diagnosis of AR was based on full clinical history, clinical examination and laboratory investigations (oesinophil count and total IgE level). Total nasal symptoms score (TNSS) (Ellis et al. 2013) comprising sneezing, runny nose and itchy nose were used to assess the clinical symptoms of AR patients, before and after treatment. All enrolled patients were non smokers. The presence of any other atopic disease or apositive family history for atopy, was recorded. Exclusion criteria included; patients aged below 15 and above 80 years old, pregnant and lactating females, patients with chronic sinusitis, patients with a history of treatment with systemic steroids in the previous 8 weeks or local steroids in the previous 4 weeks and those having any contraindication to local steroid treatment. Ten apparently healthy volunteers (5 males and 5 females) served as control individuals. Their ages ranged from 19-45 years (mean 32.5±17.7). They had no history of nasal or sinus disease, upper respiratory tract infection, AR and had received no GC treatment for at least 4 weeks prior to participating in this study. A written informed consent was obtained from all participants before their involvement in this study. The study was approved by the Institutional Reviewer Board (IRB) of Zagazig University Hospitals.

A clinical trial was conducted where all patients as well as control subjects were treated with intranasal mometasone furoate in the form of 2 puffs in each nostrils daily for 4 weeks. Nasal mucosa from all participants was obtained from the inferior turbinate before and after completion of therapy. Before obtaining the biopsy, lidocaine hydrochloride spray was administered as local anesthetic as well as xylometazoline hydrochloride to produce local vasoconstriction. All specimens were completely submerged in RNAlater Tissue Protect Tubes (Qiagen, Germany) and kept at room temperature for a maximum of one week till their examination. If further de-

Table 1. Primer sequences for both RT-PCR and quantitative real-time PCR.

Target gene	Primer sequences	Product size (bp)
GR primer used in RT PCR (Shirasaki et al. 2004)	Forward 5'-TGC AGC AGT GAA ATG GGC AA-3' Reverse 5'-GGG AAT TCA ATA CTC ATG GTC-3'	534
GRα primer used in real-time PCR (Li et al. 2010)	Forward 5'-TGAAAATGGGTTGGTGCTTCTA-3' Reverse 5'-GACAAGAATACTGGAG ATTTGAGTCAA-3'	85
β actin primer used in both reactions (Shirasaki et al. 2004)	Forward 5'-TGT GCC CAT CTA CGA GGG GTAAGC-3' Reverse 5'-GGT ACA TGG TGG TGC CGC CAG ACA-3'	100

Table 1. legend. The table demonstrates primer sequences used in both convevtional and real time PCR. The first primer targets the GR gene as a whole and it was used in conventional RT-PCR method. The second primer targets the GRα gene specifically and this was used in real time PCR method. The third primer targets the β actin gene and it was used in both methods.

lay was expected, tubes were kept at 4˚C.Total RNA was extracted from the preserved tissues using RNA-spin total RNA extraction kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. One µg of the extracted RNA was used for reverse transcription reaction using Maxime RT PreMix kit with Oligo $(dT)_{15}$ primer (iNtRON Biotechnology, Korea). RT-PCR was performed, as described previously (Shirasaki et al. 2004), to ensure the presence of DNA liable for amplification. All reactions were performed using Veriti 96-well thermal cycler (Applied Biosystems, Singapore) in a total volume of 20 µl. Maxime PCR PreMix Kit (*i*-Taq) (iNtRON Biotechnology, Korea) beads were used according to the manufacturer's instructions. Twenty µM of primers targeting both GR gene as well as beta actin gene (as an internal control) (Table 1), were used. Primers were supplied from The Midland Certified Reagent Company Inc., Texas. PCR products were examined after electrophoresis in 1.5% agarose gel and visualized under UV light. Quantitative real-time PCR was carried out to assess the expression level of GRα mRNA, before and after treatment. Beta actin gene was used as a housekeeping gene. Primer sequences (Li et al. 2010) are demonstrated in Table 1. Primers were supplied from The Midland Certified Reagent Company Inc., Texas. Reactions were performed in Stratagene MX3005P thermal cycler (Agilent technologies, Germany) using Real MOD^{TM} Green Real-time Master mix kit (iNtRON Biotechnology, Korea), in which Sybr green is the DNA detector. All reactions were performed, according to the manufacturer's instructions, in 20 µl of a mixture containing 2µl of cDNA, 20 µM of each primer and 10 µl 2X Sybr green master mix. Cycling conditions were as follows; initial denaturation for 30 sec at 95˚C followed by 40 cycles of 2 stages, the first one at 95˚C for 5 sec and a second stage at 60˚C for 30 sec. Then, a dissociation step (melting curve analysis) was done that included 2 stages; the first one at 95˚C for 15 sec and the second begins at 60˚C and ends at 95˚ for 1 min. Each sample was run in duplicate. PCR product sizes were confirmed by melting curve analysis and by gel electrophoresis. The comparative C_T ($\Delta\Delta$ C_T) method (Livak and Schmittgen 2001) was applied to calculate the relative GRα gene expression. The mean value of cycle threshold (C_T) readings of untreated AR patients and untreated healthy subjects was set as a calibrator for the patient group and the control group, respectively. The relative expression of GRα, normalized to beta actin gene and relative to the calibrator, was calculated by the following mathematical formula: $2^{-\Delta\Delta C}{}_T$. Data was analyzed using Epi-Info version 6 and Excel for windows version 8. Chi squared test was applied for comparing qualitative variables. Mann Whitney-U test was used to compare groups with not normally distributed data. The strength of the relationship between two sets of data was assessed using Spearman's rank correlation coefficient.

RESULTS

The demographic and clinical data of the studied population are presented in Table (2).The results of RT-PCR demonstrated that GR was expressed in all samples examined, whether those of AR patients or control subjects. Following 4 weeks of intranasal GC therapy, the fold change (FC) expression of GRα, calculated by $\Delta\Delta$ C_T method, was decreased in relation to the calibrator (untreated control) in 100% of healthy subjects, recording a range from 0.1 to 0.6 (mean 0.4±0.2). In 50% of them (n=5), the expression level was nearly halved (FC 0.5,

Table 2. Demographic and clinical data of the studied population

	AR patients (n=20)		Healthy subjects (n=10)		P
	No.	%	No	%	
Sex					
• Female	8	40.0	5	50.0	0.1
• Male	12	60.0	5	50.0	
Family history of atopy	5	25.0	0	0.0	0.1
Residence					
• Rural	7	35.0	2	20.0	0.4
• Urban	13	65.0	8	80.0	
Other atopic diseases	6	30.0	0	0.0	
	Mean ±SD	Range	Mean±SD	Range	P
Age (years)	31.3±8.7	18-45	32.5±17.7	19-45	0.8
Duration of illness (months)	24 ± 14.5	7-48	-	-	
Total nasal symptom score	6.6 ± 1.1	5-9	-	-	

Table 2 legend. The table demonstrates the demographic as well as the clinical data of the studied groups. It demonstrates that both groups (AR patient and healthy subjects) were matching in age, sex and residence with no significant differences detected (P 0.8, 0.1, and 0.4 respectively) between them. In addition, no significant difference (P 0.1) was found between both groups regarding family history of atopic diseases, though being positive in 25% of AR patients compared to 0.0% of healthy subjects. The table also demonstrates that the mean duration of illness in AR patients was 24 ±14.5 months and that their TNSS ranged from 5-9 befor treatment.

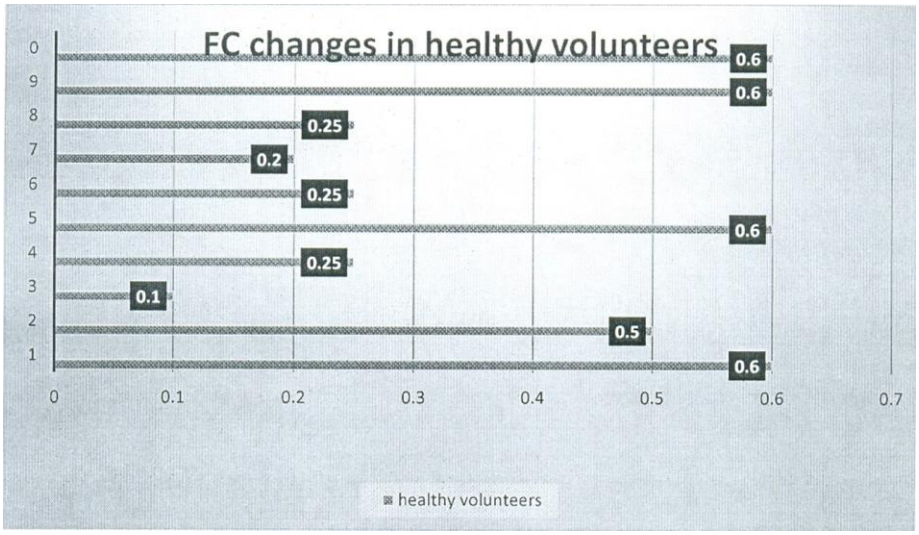

Figure1. Fold change (FC) in GRα mRNA expression after 4 weeks GC treatment in healthy volunteers (n=10)
The figure demonstrates that in 50% of healthy volunteers (n=5), the expression level was nearly halved (FC 0.5, 0.6), while it recorded one forth of the pretreatment level (FC 0.25) in 30% (n=3) of them. More decrease (FC 0.1, 0.2) was recorded in 20% of them (n=2).

0.6), while it recorded one forth of the pretreatment level (FC 0.25) in 30% (n=3) of them. More decrease (FC 0.1, 0.2) was recorded in 20% of examined subjects (n=2) (Figure 1).

Variable results were obtained, on the other hand, in the patient group (Figure 2). The expression level of GRα was downregulated in 85% of patients (n=17). In 40% of patients (n=8), a FC of less than 0.5 was recorded and a

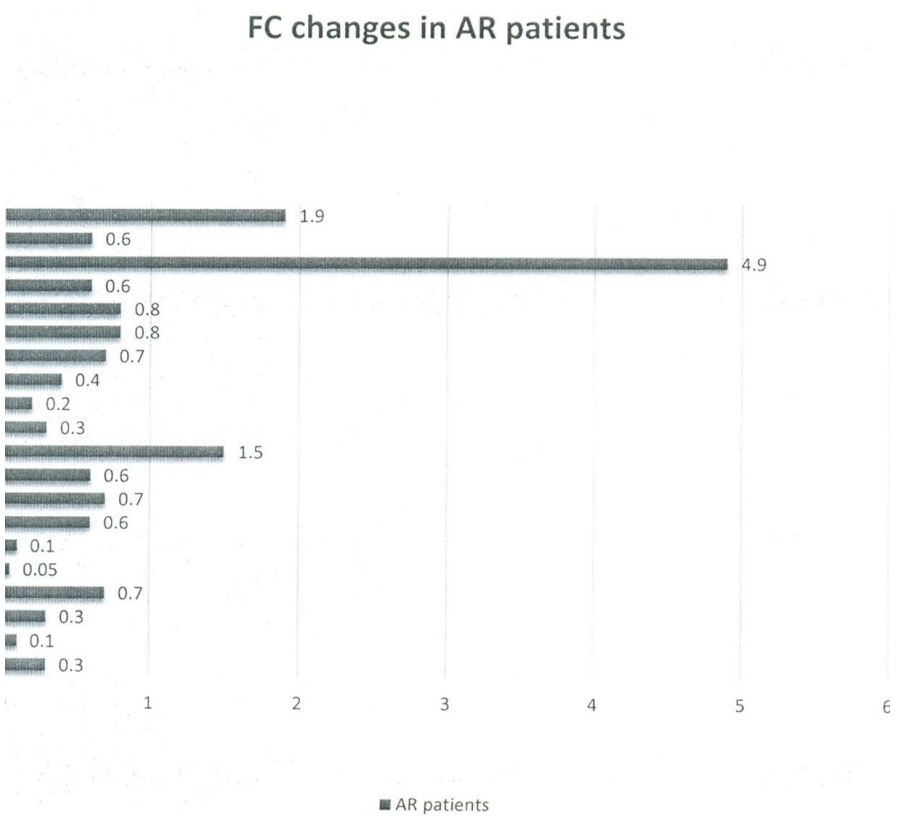

Figure 2. Fold change (FC) in GRα mRNA expression after 4 weeks GC treatment in AR patients (n=20)
The figure demonstrates that the expression level of GRα was downregulated in 85% of patients (n=17), where in 40% of them (n=8), an FC of less than0.5 was recorded and a decrease ranging from 0.5 to <1 was detected in 45% (n=9) of them. Unexpectedly, the expression was increased in 15% (n=3) of patients recording FC of 1.5, 1.9 and 4.9.

Table 3. Total nasal symptom score (TNSS) of AR patients before and after glucocorticoid treatment by Mann Whitney (MW) test

	TNSS before treatment	TNSS after treatment	MW	P	
Range	5-9	0-5		3.0	0.0**
Average	6.6 ± 1.1	1.4 ± 1.7			

** Highly significant
Table 3 legend. The table demonstrates a highly significant (P 0.0) difference between the TNSS of AR patients before and after GC therapy where it ranged from 0-5 after treatment compared to 5-9 before it. Statistical analysis of data was performed using the non parametric Mann Whitney-U test.

FC from 0.5 to <1 was detected in 45% (n=9) of them. Unexpectedly, the expression level was increased in 15% (n=3) of patients showing FC of 1.5, 1.9 and 4.9. Although the mean FC of GRα expression was higher in the patient group compared to that of the control group (0.8±1.1 compared to 0.4±0.2), this was statistically insignificant (P 0.1) when calculated by Mann Whitney-U test.

A significant (P 0.0) decrease of TNSS of AR patients was recorded after intranasal GC treatment (Table 3), which indicated good clinical response. Despite the previous finding, 65% of patients (n=13) had residual nasal symptoms and did not improve totally after treatment.

When compared, more decrease of GRα expression was noticed in patients with residual symptoms (mean FC 0.8 ± 1.3), compared to patients that were totally improved (mean FC 0.9 ± 0.6), but this difference did not show statistic significance when analysed by Mann Whitney-U test (P 0.2). In addition, the decrease in GRα expression was detected among higher ratio (92.3%) of those with residual symptoms, compared to those who

Table 4. Change in GRα expression in AR patients with total improvement and those with residual symptoms

	Totally improved patients (n=7) No. (%)	Patients with residual symptoms (n=13) No. (%)	P
Decreased expression	5 (71.4)	12 (92.3)	0.2
Increased expression	2 (28.6)	1 (7.7)	

Table 4 legend. The table demonstrates decreased GRα expression among higher ratio (92.3%) of those with residual symptoms, compared to those who showed total improvement (71.4%). Nevertheless, this was insignificant when analysed by Chi squared test (P 0.2).

showed total improvement (71.4%). Nevertheless, this was insignificant (P 0.2), as revealed by Chisquared test (Table 4).

When the change in GRα expression was correlated to clinical response of enrolled patients, our results indicated no or weak correlation (*r* -0.2, P 0.3) between the mean FC of GRα mRNA expression and the clinical response of AR patients, as indicated by Spearman rank correlation test.

DISCUSSION

Since their introduction, intranasal GC have become established as first-line therapy for treatment of AR, particularly, in patients with moderate-to-severe disease (Price et al. 2006; Bousquet et al. 2008). The wide anti-inflammatory response of intranasal GC, though complex and not completely understood, is thought to be mediated through the GR which has different isoforms, with GRα being the most predominant and active one (Pujols et al. 2007). Previous studies have demonstrated that the expression level of GR isoforms (α and β), as well as their regulation by supraphysiological doses of synthetic GC, might interfere with the response to exogenous GC (Pujols et al. 2008).

Hence, this clinical trial was conducted in order to detect the effect of 4 weeks treatment with intranasal mometasone furoate on the expression level of GRα in the nasal mucosa of AR patients, compared to healthy control subjects. A further aim was to correlate the change in the expression level with the clinical response of those patients.

In this study, GR was shown to be expressed in the nasal mucosa of all examined individuals, whether AR patients or control subjects, as revealed by conventional RT-PCR. This comes consistent with Shirasaki et al. (2004) who used immunohistochemistry in addition to RT-PCR and demonstrated the presence of GR within all cells of nasal mucosa of patients with nasal obstruction.

As the conventional RT-PCR is only a qualitative or semi-quantitative end-point detection method, fluorescent quantitative PCR was applied in the current study to enable us measure the difference in the expression level of GRα following intranasal mometasone furoate treatment in AR patients.

Our results demonstrated that GRα mRNA expression was down-regulated (mean fold change 0.4 ± 0.2) after GC treatment in 100% of healthy subjects. This comes consistent with previous *in vitro* studies that demonstrated similar results when cultured nasal epithelial cells were examined (Pujols et al., 2001). Moreover, this does agree with a previous *in vivo* study carried out by Knutsson et al. (1996) who reported that the administration of intranasal GCs to the nasal mucosa of healthy subjects resulted in down-regulation of GR mRNA expression. The same observation was also recorded when bronchial epithelial cells and alveolar macrophages were studied *in vivo* (Korn et al. 1997).

Variable results were obtained, in the current study, when AR patients were studied. Where down-regulation of GRα mRNA was detected in 85% of studied patients after GC treatment, 15% of them showed increased expression of GRα mRNA level. In their study, Henriksson et al. (2001) reported that the inflamed nasal mucosa in nasal polyps did not show down-regulation of GR mRNA following 2 months of topical corticosteroid application. This comes in contrast to our results. However, Choi et al. (2006) reported that GRα mRNA was more expressed in patients with nasal polyps, but this elevated expression was down-regulated after GC treatment which supports our finding.

In spite of the high ratio of AR patients that demonstrated down-regulation of GRα, no significant difference (P 0.1) was detected in the level of GRα expression between patients and control subjects, in the current study. This comes in contrast to Watanabe and Suzaki (2008) who reported significant lower expression of GRα mRNA in patients with nasal polyps following GC treatment as demonstrated by immunofluorescent staining and real time PCR ($P < 0.05$). However, this comes consistent with Pujols et al. (2008) who demonstrated no significant difference in GRα mRNA expression between GC-treated and non-treated patients with severe nasal polyposis.

In this work, we recorded unexpected increase in GRα expression following intranasal GC treatment in 15% of enrolled patients. Although we could not fully explain this finding, yet in the study carried out by Pujols et al.(2008),

the researchers recorded a significant temporary increase in GRα mRNA in GC-treated patients, which returned to the pretreatment level 12 weeks after treatment. As our clinical trial lasted for 4 weeks, we could assume that this elevated expression might be a temporary condition, but this warrants further studies.

To date, the previous studies were involving patients with nasal polyps, which made the comparison between the results of our study and theirs somewhat difficult. The difference in the type of GC used in different studies, method of administration (oral and intranasal in Pujols et al. *vs* intranasal in ours) and different durations (12 weeks in Pujols et al. *vs* 4 weeks in ours), may explain these inconsistent results.

As expected, AR patients showed a significant (P 0.0) decrease of their TNSS following intranasal GC treatment, in the current study. The same finding was reported previously, where in a multicentre, double-blind, placebo-controlled, parallel group study, Fokkens et al. (2007) found the use of GC nasal spray in AR patients to be significantly effective in releaving both nasal and occular symptoms (P < 0.001 & 0.001, respectively). Furthermore, Meltzer et al. (2010) demonstrated in a double-blind, 4 week study, that the use of mometasone furoate was accompanied by significant improvement of morning and evening TNSS scores of AR patients (P 0.04 & 0.01, respectively).

Despite their proven efficiency, insensitivity to GC has been reported previously in different clinical disorders, with multiple mechanisms suggested (Gross et al. 2009; Wang et al. 2011). In cases of inflamed nasal mucosa, though still a matter of debate, it was shown that down-regulation of GRα mRNA and/or increased GRβ mRNA expression might explain insensitivity to GC treatment (Li et al. 2010).

In the current study, we found no correlation between the change in GRα expression and the improvement in patients' symptoms after treatment as indicated by their TNSS (*r* -0.2, P 0.3). Moreover, no significant difference was found in GRα expression following intranasal GC treatment in patients with total improvement compared to those with residual symptoms (P 0.2). This finding comes in agreement with Pujols et al. (2008) who did not find any correlation between the changes in GRα or GRβ expression and the improvement of nasal symptoms in patients with nasal polyps, following GC treatment. However, this comes in contrast to previous reports addressing a significant correlation (P < 0.05) between decreased GRα expression and GC resistance in another immune disorder (immune thrombocytopenia) (Ma et al. 2013).

To our knowledge, few previous published data demonstrated the relationship between GR expression and GC insensitivity in AR patients (Luo et al. 2007; Luo et al. 2014).

In their study, Luo et al. (2007) used semi-quantitative RT-PCR to reveal the relationship between the expression of α- and β- isoforms of GR, in PBMC, and response to GC in AR patients. They revealed no differencein GRα expression, between GC-sensitive and GC-resistant group. This supports the findings of our study. On the other hand, they reported a significantly higher level of GRβ expression in the GC-resistant group, compared to the sensitive one (P < 0.01). In a more recent study, Luo et al. (2014) determined the expression of GRα and GRβ in the nasal mucosa of AR patients following steroid treatment. Their results demonstrated that GRβ was significantly higher in steroid-resistant group (5.62±1.28 X102 copies/μg) compared to steroid-sensitive group (4.62±0.48 X 102 copies/μg) (P < 0.01). Furthermore, a significant difference was found in the expression ratio of GRα to GRβ between both groups, where lower ratio was recorded in steroid-resistant patients compared to steroid-sensitive patients (525.7±68.1 compared to 658.32±65.16) (P < 0.01). Taken together the previous two studies, along with our results, it appears that the three studies reported no significant role of GRα alone in the insensitivity to GC, which still warrants further studies.

The small number of enrolled patients and healthy volunteers, in our study, might be a limiting factor. In addition, the choice of one GR isoform (GRα) to be tested did not allow us to assess the possible relation to other isoforms. Yet, this might be a starting point opening the door for further researches.

CONCLUSION

In conclusion, the present study demonstrated variable GRα expression levels following 4 weeks of intranasal GC treatment in AR patients, which was not correlated to the change in patients' clinical response. Further well planned, prospective studies are needed to reveal the relationship of other GR isoforms with the therapeutic effect of intranasal GC in AR patients.

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The authors declare that this study had no fund

AUTHOR CONTRIBUTIONS

Dr. Samir Sorour Sorour contributed to the design and

supervision of the study.

Dr. Nasser Nageib Mohammed contributed to analysis and interpretation of data as well as revising the final version to be published.

Dr. Marian Asaad Gerges contributed to the performance of laboratory technical work of the study, statistical analysis of data and editing the manuscript.

Dr. Boles Samir Sabry contributed to the selection of study subjects, specimen collection, and clinical evaluation of patients before and after treatment as well as for editing the manuscript.

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