RESEARCH PAPER

Accepted 12 January 2003
Revised 26 December 2002
Received 6 May 2002
Indian J. Pharm. Sci., 2003, 65(2): 171-175

# Effect of Norgestrel on Blood-Lipid Constituents in Relation to its Biological Activity

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**Having considered the lipophilicity of norgestrel (log P=3.31) an important contributor to its action mechanism, interaction of the drug with total lipids of blood have been investigated using phospholipid binding, fatty acid composition and peroxidation phenomena as the parameters under investigation. The objective was to derive an insight into the pharmacodynamic behavior of the drug. Significant loss of phospholipid along with changes in fatty acid composition was observed after incubation of whole blood with norgestrel (60 ng/ml, contraceptive concentration in blood). This may be ascribed to binding affinity of norgestrel with lipid constituents in blood. Lipid binding affinity of the drug may have a role in the mediation of its therapeutic effect. Lipid peroxidation induction potential of norgestrel has been quantitatively measured in the context of its toxicity. The results reveal that norgestrel caused significant extent of lipid peroxidation. Ascorbic acid, an antioxidant, could significantly reduce norgestrel-induced lipid peroxidation.**

Interaction of a drug with blood-lipids correlates well with the partition coefficient parameter[1] of the drug and the biological responses[2], either therapeutic or toxic. These responses involve a chain of physicochemical events within the biophase at the receptor site[3] where the drug binds through hydrophobic and/or other types of interactions. The changes occurring in the cell membrane during partitioning of a drug in various compartments include changes in membrane constituents[4], such as, i) binding with phospholipids of biomembrane, ii) changes in the fatty acid (FA) composition and iii) lipid peroxidation.

FAs are important constituents of biomembrane that control membrane fluidity[5] and are precursors of prostaglandins and other eicosanoids that regulate important body functions[6]. Lipid peroxidation is a molecular mechanism of cell injury bringing about several changes in structures and functions of biological membranes with potential injurious consequences[7] and seems to play a role in the mediation of some pathological states[8-10]. Binding of a drug with phospholipids has been linked to its therapeutic effects[11-13]. Hence, change in the lipid pattern seems to be highly important contributor to drug action mechanism[4].

In the ongoing effort of the authors to explore drug-lipid interactions[11-25], the present investigation has been designed to explore any possible relationship existing amongst the biological responses (both therapeutic and toxic) of a steroidal progestenic compound, norgestrel (NG), with its partition coefficient and the blood-lipid pattern changes. NG, a 19-norprogestin, is widely used as oral contraceptive. It has good binding affinity to progesterone and androgen receptors[26,27] and also has considerable effect on lipid profile[28]. Considering high lipophilicity of NG, its effects on blood-lipid constituents have been studied taking phospholipid binding, fatty acid composition and lipid peroxidation as the parameters under investigation and goat whole blood (model chosen due to its easy availability and close similarity in constituents to human[29]) as the lipid source. The objective was to draw an insight into pharmacodynamic behavior of the drug from drug-lipid interaction study. Attempt has been made to study the potential of ascorbic acid (AA) as possible suppressor[20-23] of NG-induced lipid peroxidation.

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## MATERIALS AND METHODS

The study has been performed using goat whole blood as the experimental model. Blood was collected from the jugular vein of female goat (*Capra capra*) (aged 8-10 m) as per the requirement for determination of lipid loss, fatty acid composition and lipid peroxidation parameters. Authentic samples of fatty acid methyl ester (FAME) were obtained from Centre for Biochemical Technology, CSIR, New Delhi and estimation of these FAMEs was carried out at Regional Sophisticated Instrument Centre, Bose Institute, Kolkata. Pure drug sample of norgestrel was provided by Wyeth Laboratories Ltd., Mumbai.

Goat blood containing anticoagulant was incubated at 15° under nitrogen atmosphere. Both the control and drug treated blood samples were incubated for 24 h (biological half-life 16±8 h[30]) and determination of various parameters mentioned above was performed at 2nd, 5th, 8th and 24th h. For phosphorus content and lipid peroxidation estimations in a given animal set, five replicate determinations were made for each sample (control or drug-treated) at each incubation period. For fatty acid determination one measurement was done in each of control and drug treated samples at each incubation period under a given animal set.

Total lipids were extracted from blood according to the method of Bligh and Dyer[31], with methanol-chloroform mixture (2:1 v/v) and the phosphorus content was estimated according to the procedure of Allen[32], which involves the use of amidol (2,4-diaminophenol hydrochloride) in sodium bisulphite as reducing agent and ammonium molybdate (8.4%) as the color developing reagent. The color developed was measured in a spectrocolorimeter (EC model GS 5700B) at 680 nm. The content of phosphorus present in the phospholipid of whole blood was calculated from the standard curve ($r^2$ = 0.994).

Saponification of whole blood-lipid was done with methanolic sodium hydroxide and the free fatty acids were converted to their corresponding methyl esters using methanolic hydrochloric acid (2.5%) according to the method of Kates[33]. The FAMEs were first purified by TLC using n-hexane, diethyl ether and glacial acetic acid mixture as the solvent system. Then quantification of the purified FAMEs was done by GC (Pye Unicam model 104) equipped with a FID and connected to a SP 4270 integrator. FAMEs were identified using standard methyl palmitate and methyl stearate as primary standards, and comparing the relative retention time of the sample peaks with those of standard FAMEs separated on the same column under identical operational condition.

Quantitative determination of lipid peroxidation end product, i.e., TBA (thiobarbituric acid)-titres was done by the method of Tarladgis *et al.*[34] with some modification[35]. The method of measurement[34] of TBA-titres involved precipitation of the protein part of whole blood by treating with 10% trichloroacetic acid solution and centrifugation at 3000 rpm for 30 min followed by filtration of the supernatant. The filtrate was then treated[35] with 0.002 M TBA solution and the resultant mixture was boiled for half an hour. Then it was cooled to room temperature and its absorbance was estimated at 530 nm against a TBA blank [prepared from equal volumes of TBA solution and distilled water] using a spectrocolorimeter (EC model GS 5700B). The amount of lipid peroxide products was calculated from the standard curve ($r^2$=0.9807) prepared using 1,1,3,3,-tetraethoxypropane and the TBA reagent according to the methods of Tarladgis *et al.*[34]. Lipid peroxidation induced by the drug and the protective role of AA in reducing the drug-induced lipid peroxidation were determined and compared with control samples.

In all these experiments, NG was used at contraceptive concentration of 60 ng/ml[36]. To suppress NG-induced lipid peroxidation, AA was used at two equivalent human dose levels of 250 mg and 500 mg (28.4 μmol/ml and 56.8 μmol/ml of blood concentrations, i.e., effective antioxidant concentrations[37]).

## RESULTS

The results supported with statistical analysis by 't' test are shown in figs. 1, 2 and 3. Fig. 1 shows the relative percentage reduction of inorganic phosphorus content in goat blood-lipid treated with NG in comparison to control. The control samples did not show any significant change in phosphorus content in different time periods. The results show that the average loss of phosphorus in whole lipid due to drug is significant throughout the experiment. This may be accounted due to phospholipid-binding capacity of the drug.

The relative percentage changes with respect to control in FAMEs of total lipids due to drug effect are presented in fig. 2. Table 1 depicts the results supported by analysis of variance (ANOVA) and multiple comparison analysis using least significant different (LSD) procedure[38]. There is increase of saturated fatty acid (SFA) content *vis-à-vis* decrease of unsaturated fatty acids (UFA) (viz. mono (MUFA), di (DUFA) and poly (PUFA) unsaturated fatty acid) content after drug treatment.

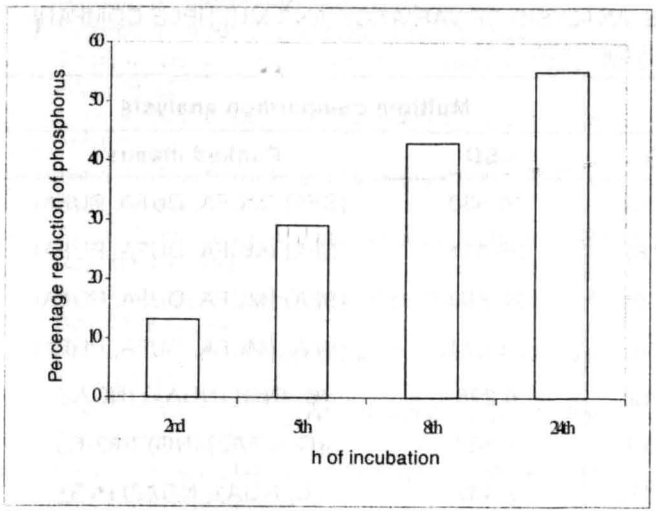


**Fig. 1: Average percentage reduction of phosphorus in blood-lipid due to norgestrel.**

**Each bar is a mean of 5 animal sets (each animal set represents average of five determinations) with standard error. Statistical comparison was made between test and control values by 't' test and the values are significant at $P \leq 0.05$.**

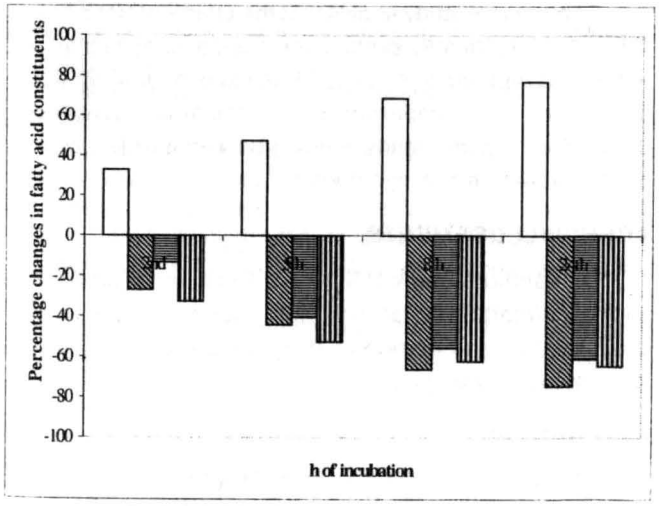


**Fig. 2: Average changes in fatty acid constituents of blood-lipid with norgestrel.**

**SFA ( ☐ ) (n=6) stands for $C_{12}$, $C_{14}$, $C_{16}$, $C_{18}$, $C_{20}$, $C_{22}$ saturated fatty acids. MUFA ( ▨ ) (n=3) stands for $C_{16}$, $C_{18}$, $C_{20}$ monounsaturated fatty acids. DUFA ( ▤ ) (n=4) stands for $C_{16}$, $C_{18}$, $C_{20}$, $C_{22}$ diunsaturated fatty acids and PUFA ( ▥ ) (n=7) stands for $C_{18:3}$, $C_{20:3}$, $C_{18:4}$, $C_{20:4}$, $C_{22:4}$, $C_{20:5}$, $C_{22:5}$ polyunsaturated fatty acids. Each bar is a mean of 3 animal sets with standard error. Probability levels ($P$) of changes are significant at a≤0.05 and b≥0.05.**

Fig. 3 represents the relative percentage changes of TBA-titre due to effect of drug alone and also in combination with AA in two concentrations. The results show that peroxide end products (in terms of TBA-titre) in NG-treated blood increase significantly in comparison to control in a time-dependent manner. Coadministration of AA significantly suppresses drug-induced lipid peroxidation.

## DISCUSSION

The results reveal that NG appreciably lowers the phosphorus content of the blood-lipid, which may be due to binding with phospholipids. This observation corroborates the fact that because of its high lipophilicity (log P=3.31), the drug diffuses passively through cellular lipoidal membrane and may bind[3,39] to a progesterone receptor (PR) and/or androgen receptors (AR). Biologically active PRs are present in the nucleus of the target cells[40]. The PR is a ligand-activated nuclear transcription factor that binds progesterone with high affinity, resulting in changes of the steroid-receptor complex, which interacts with a progesterone response element in target genes, and initiates transcription of the DNA sequence and regulate their expression[41]. NG may bind to the PR with hydrophobic bonding, resulting in decreases in estrogen receptor levels that blunt cellular responses to

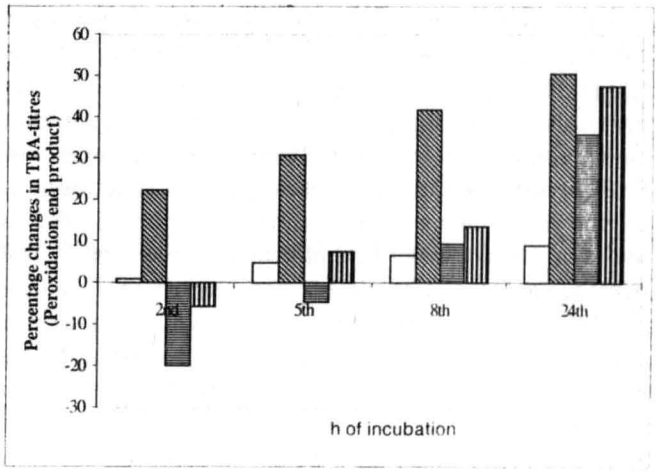


**Fig. 3: Average percentage changes in TBA-titres induced by NG, NGA1 and NGA2.**

**C ( ☐ ), NG ( ▨ ), NGA1 ( ▤ ) and NGA2 ( ▥ ) indicate control, norgestrel treated, norgestrel and ascorbic acid treated (in two dose: 250 mg and 500 mg equivalent human dose levels respectively) samples respectively. Each bar is a mean of 5 animal sets (each animal set represents average of five determinations) with standard error. Probability levels ($P$) of changes are significant at a≤0.05 and b≥0.05.**

TABLE 1: STATISTICAL ANALYSIS OF THE OBSERVATIONS: ANALYSIS OF VARIANCE AND MULTIPLE COMPARISON ANALYSIS OF DATA

| Ref. Figure | h of incubation | ANOVA * | | Multiple comparison analysis | |
|---|---|---|---|---|---|
| | | $F_1$ (df) | $F_2$ (df) | LSD* | Ranked means# |
| Fig. 2 | 2nd | 16.20[a] (3,6) | 1.69[b] (2,6) | 25.530 | (SFA) (MUFA, DUFA, PUFA) |
| | 5th | 38.05[a] (3,6) | 1.32[b] (2,6) | 26.311 | (SFA) (MUFA, DUFA, PUFA) |
| | 8th | 98.97[a] (3,6) | 5.09[b] (2,6) | 22.658 | (SFA) (MUFA, DUFA, PUFA) |
| | 24th | 130.65[a] (3,6) | 6.61[b] (2,6) | 21.814 | (SFA) (MUFA, DUFA, PUFA) |
| Fig. 3 | 2nd | 75.28[a] (3,12) | 0.56[b] (4,12) | 6.228 | (C, (NG) (NGA1) (NGA2) |
| | 5th | 262.62[a] (3,12) | 0.92[b] (4,12) | 2.867 | (C, NGA2) (NG) (NGA1) |
| | 8th | 44.91[a] (3,12) | 1.15[b] (4,12) | 7.462 | (C, NGA1, NGA2) (NG) |
| | 24th | 76.60[a] (3,12) | 2.18[b] (4,12) | 6.667 | (C, (NG, NGA2) (NGA1) |

SFA, MUFA, DUFA and PUFA stand for saturated, monounsaturated, diunsaturated and polyunsaturated fatty acids respectively. C, NG, NGA1 and NGA2 indicate control, norgestrel treated and norgestrel and ascorbic acid (in two dose levels: 250 and 500 mg respectively) treated samples respectively. Significance levels of *F* values: [a]$P \leq 0.05$ and [b]$P \geq 0.05$. * Represents analysis of variance in two ways - $F_1$ (between samples) and $F_2$ (between animal sets), df indicates degree of freedom. *Denotes least significant difference procedure[38] (critical difference at 5% level). #Indicates two means not included in same parentheses are statistically different at $P \leq 0.05$.

estrogenic agents[36] and thereby suppress uterine proliferation and exert contraceptive effect. But when NG binds with AR, it causes intrinsic androgenic effect[42] that may be related to the metabolism of lipid[43]. The changes in fatty acid composition due to drug effect are in good relation with binding of drug with phospholipid[5]. Decrease in UFA (especially PUFA) level due to drug effect may be correlated with increase in lipid peroxidation end products because PUFAs are good substrates for lipid peroxidation process[44,45]. This decrease in PUFA level may be responsible for toxic effects of NG. As lipid peroxidation is a molecular mechanism of cell injury with potential injurious consequences[44], increase in peroxide end products due to NG may be related with its toxic potential. Thus the replacement therapy of progestin with norgestrel increases the risk of venous thromboembolism[46,47] and other side effects[36] which may result from generation of toxic peroxidation end products.

The observations also show that presence of AA significantly suppresses the drug induced lipid peroxidation and AA at equivalent human dose level of 250 mg has better antiperoxidative effect than the higher dose level. This suggests that AA at lower dose level may have potential of reducing drug-induced toxicity on coadministration with drug. The increase in lipid peroxidation in AA treated sample at higher dose may be due to the prooxidant[48] effects.

The present study shows that the effects of NG on blood-lipid constituents may explain some aspects of its biological action (therapeutic and toxic). Thus study of drug-lipid interaction may be an important tool in explaining dynamics of drug action. Further study is going on with a wider variety of drugs to confirm this hypothesis.


## ACKNOWLEDGEMENTS

The authors thank UGC, New Delhi, India for financial support, Wyeth Laboratories Ltd., Mumbai, India for pure drug sample, and Pomona College, USA for supplying the log P value of the drug.


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