NORMAL DELIVERY INDUCED STRESS ALTERS GLUTATHIONE PEROXIDASE AND TNF- α IN ELDERLY PRIMIGRAVIDAS MONONUCLEAR CELLS

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ABSTRACT

To probe the glutathione peroxidase (GPx) activity coupled to ROS and TNF- α levels in younger versus elderly primigravidas. Monocytes were isolated from blood of young and elderly primigravidas after 12 hrs, 24 hrs and 14 days postpartum. Age-matched healthy female donors served as controls. Thereafter, the above monocytes were subjected to evaluation of GPx activity, levels of pro-inflammatory cytokine TNF- α and reactivity/specificity towards anti-TNF- α antibody. Suppression in GPx activity with simultaneous enhancement of TNF- α levels in monocytes was observed after 12 hrs and 24 hrs of spontaneous labor pain-induced maternal delivery through normal vaginal route. Conversely, an enhancement of GPx activity and decrease in TNF- α levels were observed on day 14 of postpartum, thereby indicating reversal of the impaired neutralizing mechanisms. The antioxidant network combating ROS was stronger in younger primigravidas. Capability of TNF- α and ROS suppression was somewhat lesser in elderly primigravidas even 14 days of postpartum via normal vaginal route. The capability of GPx activity enhancement with simultaneous suppression in ROS and TNF- α , decreases in elderly primigravidas. This may act as one of the important parameters leading to a variety of complications encountered by elderly primigravidas.

KEY WORDS

Tumor Necrosis Factor-alpha (TNF- α), Monocytes, Glutathione peroxidase.

INTRODUCTION

Humans under stress of any kind are known to generate free radicals like reactive oxygen species (ROS) and reactive nitrogen intermediates (1, 2). The function of antioxidant enzymes is to protect cells from toxic oxygen. During pregnancy, oxidative stress has also been associated with reproductive problems. It is well documented that oxygenation of both maternal and fetal tissue oscillate frequently during labor (3). Maternal oxygen consumption increases significantly in normal labor (4). Documented reports show that there are often periods of apnea and/or shallow respiration between

uterine contractions (5). Also, uterine contractions are known to cause reduction in uterine blood flow (6). These evidences strongly indicate the generation of tremendous amount of ROS during labor, and that; maternal age may be an important factor where women encountering late age primarygravidia i.e. age nearing menopause, may encounter complications during advanced stage than normal term pregnancies. As a matter of fact, only scattered information is available about baseline levels of antioxidants and pro-inflammatory cytokine TNF- α in healthy pregnant women, particularly in postpartum. Although several reports are available where workers have described and quantified the maternal and fetal response to pain and stress of labor, there is no information about the relationships between glutathione peroxidase (GPx) and pro-inflammatory cytokine TNF- α with comparative clinical characteristics between early age and late age women undergoing stress by labor pain following normal delivery.

In the present study, keeping in mind about the high intensity of pain during labor (non-epidural) leading to child birth, where

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patient is under a high magnitude of natural stress, we probed the status of antioxidant enzyme GPx and TNF- α , a known pro-inflammatory autocrine cytokine, in women of age group between 20-40 years after 12 hrs, 24 hrs and 14 days of normal vaginal route of delivery. Since it was difficult to keep a track and follow-ups in order to obtain samples from the same patients after 14 days of delivery, thus day 14 was the cut-off point for getting samples in this study. Also, age after 40 years was not included in this study as this is the normal onset age of menopause. It is hoped that the data generated may help in the better understanding about the difference at the molecular level between early age and late age women delivering babies via normal vaginal route.

MATERIALS AND METHODS

Ficoll-Paque was obtained from Pharmacia (LKB biotechnology Piscataway, NJ). ELISA Kit for measurement of TNF- α was purchased from R&D Systems. All other chemicals used were of the highest purity grade available. ELISA microtitre plates were read on automated microplate ELISA Reader (Qualigens, India).

Patients : Blood obtained from healthy female donors (n-5 from each age group) were used as control. Blood comprising test samples were collected from patients after taking prior consent in the age group of 20-40 yrs (n-6 from each age group; total 18 patients), at the end of third trimester, labor, at the time of delivery, 12 hrs, 24 hrs and 14 days post partum. The age groups were divided into four categories, which were

20-25 yrs as category I, 26-30 yrs as category II, 31-35 yrs as category III and 36-40 yrs as category IV. These patients were regular O. P. D. patients, who were in turn admitted for delivery in J. N. Medical College and Hospital, AMU, Aligarh. All patients belonged to middle-income group with no known history of allergies. Their maternal and fetal weights were recorded in the range of 45-65 kgs and 2.6 to 3.6 kgs respectively. Except for prenatal vitamins and iron supplements in pregnancy, they were not under any kind of medication for any disease. Furthermore, the selected patients were non-smokers and were free from any pregnancy complications or any maternal or amniotic infections. The usual length of labor was in between 8-12 hrs, and all were full term normal deliveries.

Isolation and sonication of mononuclear cells : Separation and isolation of mononuclear cells from whole blood was carried out as described earlier (7). Thereafter, the cells were subjected to sonication on ice at 4 output (60% duty cycle) for 2 min in 20 seconds bursts. The extracts were centrifuged at maximum speed for 20 min at 4°C. Supernatant obtained was used as enzymatic and pro-inflammatory cytokine TNF- α extract.

Monitoring the status of glutathione peroxidase (GPx) in mononuclear cells : GPx activity in control and test samples was determined as described by others and us previously (7, 8).

Inhibition ELISA : Antigenic specificity of anti-TNF- α antibodies was determined on flat bottom 96-well polystyrene

Table 1 : Determination of glutathione peroxidase (GPx) activity

[A] GPx activity (U/mg protein) in control mononuclear cells of blood obtained from healthy non-pregnant females and during pregnancy (3rd trimester) in age groups of 20-40 yrs

Age Grp. (yrs)	Control non-pregnant	3 rd trimester pregnancy
20-25 yrs (category I)	72	71 U/mg protein
26-30 yrs (category II)	72	71
31-35 yrs (category III)	63	63.2
36-40 yrs (category IV)	58	59

[B] GPx activity (U/mg protein) in mononuclear cells of blood obtained during 6-10 hrs labor, delivery, 12 hrs postpartum (PP), 24 hrs postpartum (PP) and 14 days postpartum (PP) in age groups of 20-40 yrs.

Age Grp. (yrs)	Labor	Delivery	12 hrs PP	24 hrs PP	14 days PP
20-25 yrs (category I)	39	36	38	49	66.42
26-30 yrs (category II)	39	35	38	48	66.42
31-35 yrs (category III)	35	32	30	39	58
36-40 yrs (category IV)	24	18	20	26	45

The results depicted in Table 1 are mean of six patients from each age group. GPx activity is expressed as $\mu\text{mol NADPH/min/mg protein}$ (U/mg protein). Results are Mean $\pm$ SD from triplicate experiments.

Table 2 : Determination of TNF- α by ELISA in mononuclear cells

[A] TNF- α (pg/ml) levels were determined by ELISA in mononuclear cells of blood obtained from non-pregnant and during pregnancy (3rd trimester) as well as during labor, delivery, 24 hrs and 14 days postpartum.

Age Grp. (yrs)	Non-pregnant	3 rd trimester	Labor	Delivery	24 hr PP	14 days PP
20-30 yrs (category I & II)	2.63	3.16	88.23	112.32	169.52	11.75
31-35 yrs (category III)	8.26	9.22	152.76	179.98	225.07	26.98
36-40 yrs (category IV)	12.32	13.28	181.32	239.33	290.95	38.42

Results are Mean $\pm$ SD from triplicate experiments. (n=6 for all age groups)

plates by competition ELISA as reported earlier (9).

Measurement of TNF- α in mononuclear cells : The amount of TNF- α in samples prepared from mononuclear cells of normal control and patients selected in this study was determined by use of a commercial ELISA Kit (R & D systems), according to the manufacturer's specifications. The cut off or lower sensitivity was 4.4 pg/ml (10).

Statistical analysis : Results were analyzed statistically by ANOVA. A P -value =0.05 was considered significant.

RESULTS

Determination of Glutathione peroxidase (GPx) activity in mononuclear cells : GPx activity of mononuclear cells from categories I and II non-pregnant control and 3rd trimester pregnant females exhibited GPx activities to the order of ~72 U/mg protein ($P<0.001$) and ~71 U/mg protein ($P<0.001$) respectively (Fig. 1A), which in turn, was suppressed by 13-15% and 26-30% respectively (Table 1) in categories III and IV samples. Interestingly, even non-pregnant controls showed age-dependent suppression in GPx activities (Table 1A). Furthermore, all the four categories of patients exhibited high magnitude suppression in GPx activities in mononuclear cells collected from blood during labor (n-6), delivery (n-6), 12 hrs postpartum (n-6) and 24 hrs postpartum (n-6) (Table 1B). Nearly similar significant observations ($P<0.001$) were recorded in all age groups. Interestingly, GPx activities determined during follow-up after 14 days of normal delivery exhibited an appreciable degree of reversal in GPx activities (66.42 U/mg protein, $P<0.001$) in category I and II patients (Table 1B), whereas the efficiency of GPx activity reversal after 14 days postpartum in category III and IV primigravidas were found to decrease by around 15-20% (Table 1A and 1B).

Determination of TNF- α expression in mononuclear cells of non-pregnant, during pregnancy, labor, delivery, 24 hrs and 14 days postpartum : Category I and II samples of non-pregnant controls as well as pregnant females during third

trimester exhibited negligible levels of TNF- α (Table 2), which augmented linearly in test samples during labor (88.23 pg/ml, $P>0.001$), delivery (112.32 pg/ml, $P>0.001$) and 24 hrs postpartum (169.52 pg/ml, $P>0.001$) respectively. This was followed high magnitude suppression in TNF- α expression (11.75 pg/ml, $P>0.05$) after 14 days postpartum, thereby indicating for a strong reversal of TNF- α expression to near normal control values after 14 days postpartum (Table 2). Next, a relatively high TNF- α expression in category III samples were recorded as 8.26 pg/ml ($P>0.03$), 9.22 pg/ml ($P>0.03$), 152.76 pg/ml ($P>0.001$), 179.98 pg/ml ($P>0.001$), 225.07 pg/ml ($P>0.001$) and 26.98 pg/ml ($P>0.05$) for healthy non-pregnant females, third trimester pregnant females, labor, delivery, 24 hrs and 14 days postpartum respectively (Table 2). Next, TNF- α expression in category IV samples was recorded as 12.32 pg/ml ($P>0.04$), 13.28 pg/ml ($P>0.03$), 181.32 pg/ml ($P>0.001$), 239.33 pg/ml ($P>0.001$), 290.95 pg/ml ($P>0.001$) and 38.42 pg/ml ($P>0.05$) for healthy non-pregnant females, third trimester pregnant females, labor, delivery, 24 hrs and 14 days postpartum respectively (Table 2).

Computational analysis of the above data depicted in Table 2 exhibited mean TNF- α expression to augment by around 2.06-fold, 2.13-fold, 1.72-fold and 3.27-fold in monocytes during labor, delivery, 24 hrs and 14 days postpartum respectively in category IV primigravidas in comparison to category I and II primigravidas. Similar trend with somewhat lower values were observed when compared to category III primigravidas. Thus, the results show that even after 14 days postpartum, the TNF- α expression was 3.27 fold higher in category IV elderly primigravidas in comparison to younger primigravidas.

Inhibition ELISA : Next, since the role of reactive oxygen species (ROS) and pro-inflammatory cytokine TNF- α under stress conditions is established, thus, an attempt was made to probe the labor pain stress-induced immune response against monoclonal anti-TNF- α antibody in human monocytes.

No appreciable binding was observed with monoclonal anti-

TNF- α antibody on plates coated with total protein lysates obtained from 24 hrs cultured monocytes of normal healthy non-pregnant females or pregnant 3rd trimester (Table 3 A and B), thereby indicating the levels of ROS and pro-inflammatory cytokine TNF- α was not augmented and it remained nearly the same as in healthy non-pregnant females. On the contrary, category I and II primigravidas (during 6-10 hrs labor) exhibited a maximum of ~73.51% ($P<0.001$) inhibition in monoclonal anti-TNF- α antibody activity, where

50% inhibition was achieved at an inhibitor concentration of ~0.07 ng/ml (Table 3C). Category III and IV primigravidas exhibited an augmented inhibition in anti-TNF- α antibody activity (84.72% and 87.93% respectively at 10 ng/ml inhibitor, $P<0.001$) Fifty percent inhibition for category III and IV primigravidas was achieved at inhibitor concentrations of 0.007 and 0.004 ngs/ml respectively (Table 3C). Similar binding data was recorded with total protein lysates of monocytes from blood drawn immediately after delivery (Table 3D) or 24 hrs

Table 3 : Binding Characteristics of Anti-TNF- α antibody by Inhibition ELISA

Age groups	Maximum % Inhibition in antibody activity	Maximum % Inhibition Concentration (ng/ml)	Inhibitor Concentration at which 50% inhibition in anti-TNF- α antibody was achieved
(A) Monocytes obtained from blood of non-pregnant healthy females:			
(a) 20-25 yrs	19.12% ($P<0.01$)	10 ng/ml	Not achieved
(b) 26-30 yrs	22.33% ($P<0.03$)	10 ng/ml	Not achieved
(c) 31-35 yrs	27.87% ($P<0.02$)	10 ng/ml	Not achieved
(d) 36-40 yrs	31.23% ($P<0.03$)	10 ng/ml	Not achieved
(B) Monocytes obtained from blood after 3rd trimester:			
(a) 20-25 yrs	20.02% ($P<0.01$)	10 ng/ml	Not achieved
(b) 26-30 yrs	25.12% ($P<0.03$)	10 ng/ml	Not achieved
(c) 31-35 yrs	30.44% ($P<0.02$)	10 ng/ml	Not achieved
(d) 36-40 yrs	34.98% ($P<0.03$)	10 ng/ml	Not achieved
(C) Monocytes obtained from blood during labor:			
(a) 20-25 yrs	73.51% ($P<0.001$)	10 ng/ml	0.07ng/ml
(b) 26-30 yrs	76.77% ($P<0.001$)	10 ng/ml	0.06 ng/ml
(c) 31-35 yrs	84.72% ($P<0.001$)	10 ng/ml	0.007ng/ml
(d) 36-40 yrs	87.93% ($P<0.001$)	0.1 ng/ml	0.004 ng/ml
(D) Monocytes obtained from blood immediately after delivery:			
(a) 20-25 yrs	72.32% ($P<0.001$)	10 ng/ml	0.06 ng/ml
(b) 26-30 yrs	78.22% ($P<0.001$)	10 ng/ml	0.06ng/ml
(c) 31-35 yrs	87.21% ($P<0.001$)	10 ng/ml	0.006 ng/ml
(d) 36-40 yrs	90.11% ($P<0.001$)	0.1 ng/ml	0.004 ng/ml
(E) Monocytes obtained from blood after 24 hrs of normal delivery:			
(a) 20-25 yrs	69.11% ($P<0.001$)	10 ng/ml	0.08 ng/ml
(b) 26-30 yrs	74.97% ($P<0.001$)	10 ng/ml	0.07 ng/ml
(c) 31-35 yrs	82.62% ($P<0.001$)	10 ng/ml	0.008 ng/ml
(d) 36-40 yrs	85.83% ($P<0.001$)	0.1 ng/ml	0.005 ng/ml
(F) Monocytes obtained from blood after 14 days of normal delivery:			
(a) 20-25 yrs	21.12% ($P<0.01$)	10 ng/ml	Not achieved
(b) 26-30 yrs	23.03% ($P<0.03$)	10 ng/ml	Not achieved
(c) 31-35 yrs	28.68% ($P<0.02$)	10 ng/ml	Not achieved
(d) 36-40 yrs	35.67% ($P<0.03$)	10 ng/ml	Not achieved

Results are mean of 5 patients (n=5) from each age group.

postpartum (Table 3E). Comparative analysis revealed monoclonal anti-TNF- α antibody was specifically recognizing ~10-fold more its corresponding epitopes on monocytes from category IV primigravidas than category I and II primigravidas. On the other hand, negligible binding of monoclonal anti-TNF- α antibody was observed with all the four categories of primigravidas after 14 days of post normal delivery (Table 3F).

DISCUSSION

In the present study, we observed that GPx activities were unaffected in control healthy non-pregnant age matched females as well as at the end of third trimester in pregnant females. On the contrary, GPx activities were found to decrease appreciably in monocytes obtained from blood of patients at the time of labor, delivery as well as 12 hrs and 24 hrs after delivery through the normal vaginal route. Conversely, an enhancement of GPx activities after 14 days postpartum was observed which in turn indicated reversal of the impaired neutralizing mechanisms. Surprisingly, slightly augmented GPx activities observed in patients falling under age group of 20-30 years versus patients belonging to age groups of 31-35 yrs followed by age group of 36-40 yrs strongly indicates the presence of effective anti-oxidant network combating ROS generated as a consequence of cellular activation. It seems that as a consequence of extremely high magnitude of stress encountered by patients delivering babies via normal vaginal route, their mononuclear cells undergo cellular activation. This in turn, renders the glutathione *in vivo* somewhat semi-functional or defective, and consequently rendering the cells sensitive to inflammatory cytokines such as tumor necrosis factor alpha. The elevations of TNF- α are known to activate and increase a variety of *in vivo* cellular mechanisms by stimulating NF- κ B (1,10, 11). It may be noteworthy to point out here that our data showing enhanced GPx activity after 14 days of post delivery, is strongly suggestive for the possible fact that 14 days of time is sufficient enough to arrest the cellular activation of TNF- α and ROS in monocytes by probably ameliorating glutathione by self-defense mechanisms *in vivo*, consequently resulting in the down-regulation of TNF- α expression in mononuclear cells. It is to be pointed out that in comparison to patients in between age groups of 20-36 yrs, the degree of cellular activation arrest of TNF- α and ROS was somewhat of a lesser degree in elderly primigravidas (age group: 36-40 yrs) even after 14 days of post delivery via normal route. It seems that such a phenomenon may be one of the important contributing parameters that initiates a cascade of cellular events leading to a variety of complications encountered by elderly primigravidas. However, an in-depth investigation is mandatory to convincingly conclude the above.

The above findings were having significant correlation to the data obtained from immunological studies. The inhibition ELISA data revealing an increase in the binding of monoclonal anti-TNF- α antibody with antigens in total protein lysates of monocytes obtained from blood of patients falling under the age group of 36-40 yrs after 24 hrs of post normal delivery is in accordance to the data obtained from GPx activity and TNF- α determination experiments. The negligible or insignificant binding of monoclonal anti-TNF- α antibody observed with monocyte protein lysates obtained from blood of patients falling under various age groups after 14 days of post normal delivery is in close accordance to the TNF- α and GPx activity data observed in this study.

It is well established that during labor, even in normal progress, it is known that oxygenation of both maternal and fetal tissue oscillate frequently (2). It has been suggested for the occurrence of periods of apnea and/or shallow respiration between contractions (1, 4). These aberrations and hyperventilation-induced (12) hypocarbia are probably the cause of oxygen desaturation (4). The relationship between the above evidences reported by different workers with our results where an increase in pro-inflammatory cytokine TNF- α levels coupled to a substantial decrease in GPx activities after 12 hrs and 24 hrs of normal delivery, followed by a reversal in the GPx activities and TNF- α levels after 14 days of postpartum cannot be ruled out.

In summary, the capability of glutathione peroxidase activity enhancement/ reversal to near normal values with simultaneous suppression in ROS and TNF- α decreases appreciably after 14 days postpartum in elderly primigravidas. It seems that the findings of this study may help in throwing some light for the better understanding about the difference between early age and elderly primigravidas delivering babies via normal vaginal route. Moreover, since, ROS and TNF- α plays a pivotal role in life, thus, the findings here may also throw some light for future directions in addressing the variety of complications encountered by elderly primigravidas.

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