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Original Article

Expression of thioredoxin, 8Hydroxy-deguanosine and peroxiredoxins in placental tissues

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Abstract: In this study we analyzed the expression of 8-hydroxy-deguanosine (8OHdG), thioredoxin (Trx) and peroxiredoxins (Prx) 2, 3, 4, 5 and 6 in 80 cases of placental samples representing both normal and diseased placentas. The staining was evaluated separately in the stromal, trophoblastic and vascular components of the tissues. The results indicate that during the first trimester the level of reactive oxygen species (ROS) as indicated by 8OHdG was highest in the stromal component, which was also the case for Trx, Prx3, Prx4 and Prx6. In diseased placentas stromal 8OHdG was lower in cases with chromosomal aberrations but higher in molar disease in both trophoblastic and stromal cells. In chorioamnionitis stromal prx 5 and prx6 were high. Stromal prx4 and trophoblastic prx6 and stromal and endothelial Trx were also higher in molar disease. The results show that the oxidative stress is high in placentas during the first trimester. This probably reflects the oxidation burst of the placental tissues due to development of vascularity. In diseased placentas elevated levels of Prxs were seen in chorioamnionitis reflecting the ROS stress induced by inflammatory cells. The higher values of antioxidative enzymes in molar disease are possibly due to defective placentation. All in all, the results underline the importance on ROS associated mechanisms in placental development and the function of placenta to protect the developing fetus from harmful effects of reactive oxygen species as evidenced by their abundant expression in different cellular compartments of the placenta.

Keywords: Placenta, oxidative damage, antioxidative, peroxiredoxin, thioredoxin

Introduction

Reactive oxygen species (ROS) are reactive molecules containing oxygen and harboring unpaired electron on their outer orbital [1]. They include the hydroxyl ion, superoxide anion, hydrogen peroxide and also nitrogenous compounds such as nitric oxide and peroxynitrite [1, 2]. ROSes are reactive molecules which react with cellular compounds such as lipids, proteins, carbohydrates and DNA causing cellular damage and damage to DNA [1, 3]. Antioxidant enzymes, such as superoxide dismutases, thioredoxins, peroxiredoxins and glutathione neutralize ROSes thus counteracting their harmful effect [4]. ROSes however, participate in signal transduction of cells, thus the local balance between hydrogen peroxide, nitric oxide, thioredoxins and peroxiredoxins influences many cellular functions [3, 5].

ROSes are formed during cellular respiration in mitochondria where glucose is oxidized to carbon dioxide and water thus forming 30-36 ATP molecules in the reaction [6]. As a byproduct superoxide is formed which is metabolized by MnSOD to hydrogen peroxide which then is neutralized to water by peroxiredoxins (Prx) or by the glutathione cycle [3]. ROSes may also be formed by NAPDH oxidases and some other enzymatic reactions of the cell or during hypoxia [5, 7]. When ROSes are present in excess cells suffer from oxidative stress [1, 2]. Several exogenous factors may also induce formation of ROSes and oxidative stress such as radiation, smoking and chemotherapeutic drugs [2]. In tissue sections, oxidative stress can be detected by immunohistochemistry by antibodies to nitrotyrosine, 8OH-deguanosine or 4-hydroxy-2-nonenal [8, 9].

Thioredoxin (Trx) is a small molecule of about 12 kDa [10]. There are two thioredoxins, Trx1 which is cytosolic and nuclear and Trx2 which is mitochondrial [11]. Trx contains an active thiol/disulphide site and is able to reduce peroxiredoxins and methionine sulfoxide reductases by using NAPDH in the reactions [10, 11]. Trx interacts with transcription factors harboring critical cysteine residues such as p53, AP-1, HIF-1, Nk-κβ and nrf2 and signaling molecules such as PTEN and ASK1 [11]. In fact, Trx takes part in S-nitrosylation reaction of proteins with a target of about 2000 proteins in cells [11, 12]. Trx1 and Trx2 are both antiapoptotic and influence many other cellular functions such as migration, differentiation, proliferation and inflammation by reducing target proteins [11]. The activity of Trx on the other hand is regulated by thioredoxin reductases which reduce it along with some other enzymes and proteins [11]. There are three different thioredoxin reductases in humans [11].

Peroxiredoxins (Prx) are antioxidative enzymes which neutralize ROSes such as hydrogen peroxide and peroxynitrite [13]. They can reduce over 90% of the cellular hydrogen peroxide [14]. They are abundantly present in cells and through regulation of hydrogen peroxide participate in cellular signaling influencing cell differentiation, inflammation and proliferation [13, 14]. There are six Prxs in humans [15]. Prx 1 and 2 are located in the cytosolic and nuclear compartments in the cell, prx3 is mitochondrial, and prx4 resides in ER and mitochondria [13, 15]. Prx5 is located in mitochondria, cytosol, peroxisomes and cellular nuclei and prx6 in cytoplasm, lamellar bodies and lysosomes [13, 15]. Based on cysteine residues in their protein structure prxs are divided into typical 2-Cys, atypical 2-Cys and 1-Cys peroxiredoxins [16]. Prx 1-4 belong to typical 2-Cys group [16]. They have two sulphur containing reactive cysteine sites which can be oxidized and react with similar groups of other prxs [16]. Prx5 which belongs to atypical 2-Cys group has also two reactive cysteines and these form a bond between each other in oxidation reaction [16]. Prx 6 which belongs to 1-Cys group has one reactive cysteine site and reacts with other molecules [16]. Prx knockout mice are more vulnerable to oxidative damage and develop various deficiencies partly depending on the prx in question [15]. Prx 1 and 2 knockout mice develop hemolytic anemia and die prematurely, prx 3 mice experience increased placental oxidative stress and prx 4 knockout mice have testicular atrophy due to oxidative stress [13, 15].

Like thioredoxin, prxs are upregulated by nrf2 [16]. Thioredoxin is able to reduce the Cys-SOH site of prx back to a functional form [15]. In case of abundant oxidative stress the site is hyperoxidised to Cys-SO2H which can be reduced by sulfiredoxin [13, 15]. 2-Cys prxs may dimerize and form decamers and multiple decamers which function as chaperones [13]. Prxs are also involved in cellular circadian cycles [13, 17].

In this study we analyzed expression of trx, 8OHdG, and prx 2-6 in a set of placental tissues representing different diseases comparing them with normal placental. Expression of these ROS associated molecules were also assessed during the three trimesters of pregnancy.

Materials and methods

The material consisted of 80 cases of placental tissue of which 24 originated from the first, 27 from the second and 29 from the third trimester. In 16 cases, the placental tissue was normal. Diseased placentas included 13 with chromosomal aberrations, 13 molar diseases (three of partial moles were confirmed by cytogenetics, in the other cases the diagnosis was conducted by histopathology, elevated HCG-value and ultrasound findings), 10 cases of toxemia, 7 cases of diabetes, 18 cases of infections (placentas with infection all had chorioamnionitis) and 3 cases of placenta accrete. All the samples had been fixed in 10% neutral formalin and embedded in paraffin. During the selection of the cases, the diagnosis were re-evaluated from the hematoxylin-eosin stained sections by an experienced pathologist (EP). The study was approved by the Ethical committee of the Northern Savo Hospital District.

Immunohistology

The immunohistochemical (IHC) procedure was as follows. Four-micron thick sections were cut from the selected He-stained blocks. The sections were then deparaffinised in xylene and rehydrated in descending ethanol series. For antigen retrieval, the sections were incubated

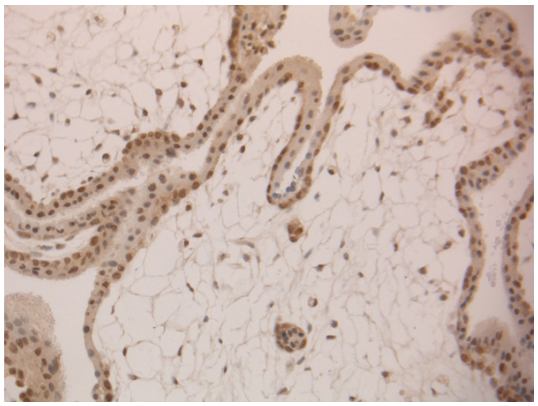

Figure 1. Trx 1 staining in first trimester placenta. Trophoblastic cells are positive but many stromal cells show nuclear positivity.

in 10 mM citrate buffer (pH 6.0) in a microwave oven for 2 minutes at 850 W followed by 8 minutes at 350 W. Endogenous peroxidase activity was blocked by incubation in 0.1% hydrogen peroxide in absolute methanol for 10 minutes. Polyclonal rabbit anti-human peroxiredoxin antibodies (Lab Frontier, York, UK) were used with a dilution of 1:1000 for Prx 2, 1:750 for Prx 3 and Prx 4, and 1:2000 for Prx 5 and Prx 6. The primary antibodies for Prxs were revealed using the Histostain-Plus Kit (Zymed Laboratories Inc., South San Francisco, CA) as described previously [18].

The primary mouse Anti-8-OHdG (MOG020P) antibody was used with a dilution of 1:50 (JaICA, Nikken SEIL Co., Ltd, Fukuroi Shizuoka, Japan) and goat anti-thioredoxin (705) with a dilution of 1:1000 (American Diagnostica, Inc., Stamford, CT, USA). The primary antibody incubations were done in a humidity chamber at +4°C overnight. For 8OHdG the Histostain-Plus Bulk Kit, LAB-SA Detection System (Invitrogen Corporation, CA, USA) was used and for thioredoxin Vectastain Elite ABC Kit (goat IgG) (Vector Laboratories Inc., CA, USA) was used. After being counterstained with hematoxylin, the slides were dehydrated and mounted with Histomount (National Diagnostics, New Jersey, USA). Previously known positive control samples were used in each series of staining. In negative controls, according to the detection system used, PBS or TBS replaced the primary antibody.

The stainings were reviewed and analyzed on a multihead microscope by two observers blinded from the clinical data. The analysis was done according to the percentage of stained cells. The cut-off point for negativity was set at 5% of the cells stained and the negative slides were marked as 0. The positive slides were divided into three groups; when 6-25% of the cells were positive the case was graded as 1, 26-50% positivity was graded as 2, 51-100% as 3.

The immunoreactivity was assessed separately in trophoblastic, endothelial and fibroblastic cells of the villi. The evaluation was performed from one representative section by evaluating the whole section area.

Statistical analysis

All statistical analyses were done using SPSS 11.0 for Windows (Chicago, IL). The significance of associations was defined using chi-square test and Fisher's exact test. Values <0.05 were considered statistically significant.

Results

Thioredoxin and 8OHdG

Thioredoxin was more commonly expressed strongly in stromal cells of the villous structures in the first trimester compared to the others (P<0.001, chi-square). (**Figures 1** and **2**). Separately, cases of the third trimester and second trimester had a lower (<2) thioredoxin expression (P=0.00003, P=0.01, respectively, chi-square). Such association was not seen for endothelial or trophoblastic expression (P= 0.108 and P=0.199, respectively, chi-square). Similarly to this 8OHdG expression was more strongly expressed in villous stromal cells in the first trimester (P=0.025, chi-square) (**Figure 3**) with also trophoblastic cells showing a similar tendency (P=0.092, chi-square) while endothelial cells did not (P=0.333, chi-square). Separately, cases in the third or second trimester did not show statistically significant difference compared to the first trimester in villous stromal reactivity for 8OHdG (P=135, P=0.123, respectively, chi-square). In general stromal thioredoxin expression and 8OHdG expression associated with each other (P=0.003, chi-square). This was also true for trophoblastic (P=0.029, chi-square) but not for endothelial positivity (P=0.086, chi-square).

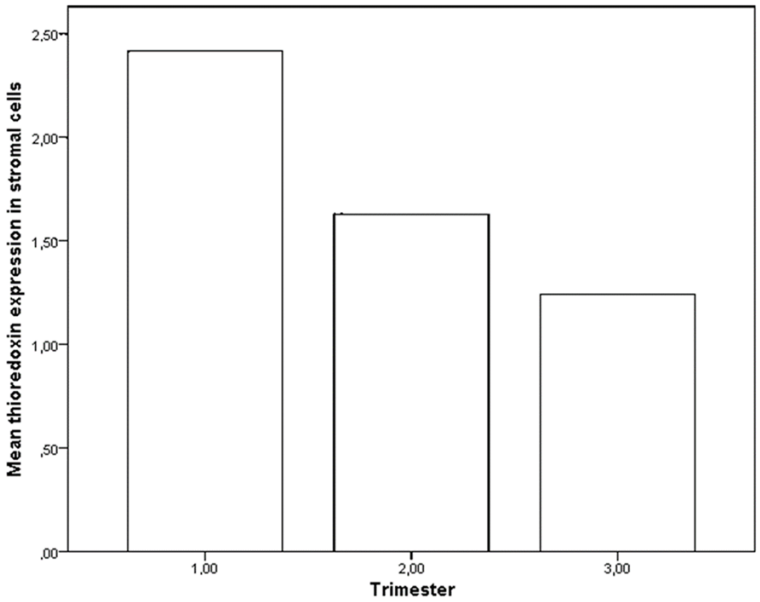

Figure 2. A graphic presentation of thioredoxin positivity in stromal cells during the three trimesters. Thioredoxin expression is highest during the first trimester followed by the second and the third.

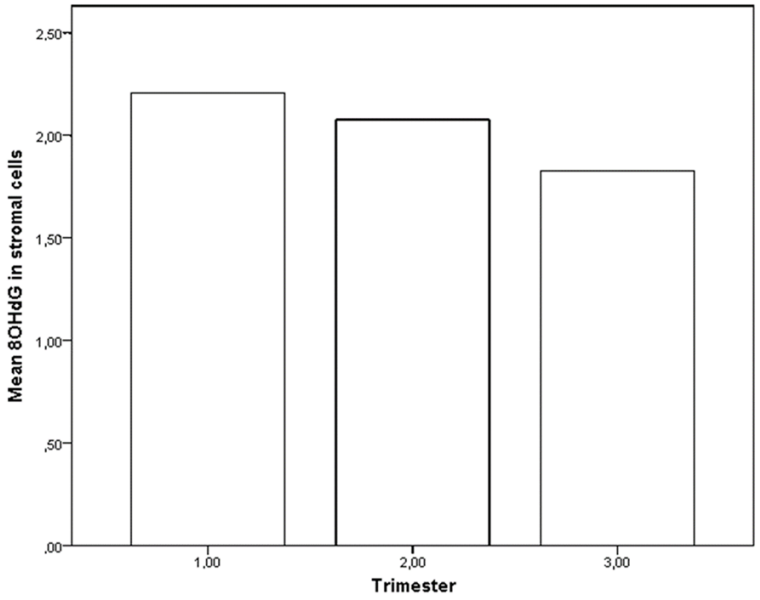

Figure 3. A graphic presentation of 8OHdG staining during the three trimesters showing a similar tendency as with thioredoxin.

Cases with aberrant chromosomal structures had a lower expression of 8OHdG in trophoblastic cells (P=0.021, Fisher's exact test). In molar disease stromal and endothelial thioredoxin expression (P=0.006, P<0.001, respectively, Fisher's exact test) were stronger and this was also case for stromal 8OHdG (P=0.001, Fisher's exact test), and trophoblastic (P=0.003, Fisher's exact test) expression but not for endothelial (P=0.157, Fisher's exact test). In diabetic placentas less thioredoxin was observed in endothelial cells (P=0.023, Fisher's exact test) while toxemic placentas or placentas with chorioamnionitis did not differ significantly from the others. Placenta accretes showed less stromal (P=0.009, Fisher's exact test) and trophoblastic (P<0.001, Fisher's exact test) thioredoxin than other cases.

Staining with peroxiredoxins

Stromal prx6 staining associated with stromal prx5 (P<0.001, chi-square) but not with stromal prx4 (P=0.245, chi-square) but stromal prx5 and prx4 (P=0.004, chi-square) and stromal prx5 and prx2 (0.012, chi-square) showed a positive association. In trophoblastic cells prx6 staining associated with prx5 and prx4 (P=0.004, P=0.017, respectively, chi-square) and prx5 with prx4 and prx2 (P< 0.001, P=0.014, chi-square). In endothelial cells prx6 staining associated with prx5 and prx4 (P=0.009, 0.034 respectively, chi-square), prx5 with prx4 (P=0.001, chi-square) and prx4 with prx2 (P=0.026, chi-square).

Stromal prx6 was stronger in the first trimester compared with the second and third trimester period (P=0.005, chi-square). This was also the case with prx4 (P=0.012, chi-square) and prx 3 (0.014, chi-square) but not with prx5 (P=0.25, chi-square). On the other hand, trophoblastic expression of prx5 was more strong in the first trimester (P=0.004, chi-square) and this was also the case for prx4 and prx3 (P=0.016 and P=0.009, respectively, chi-square). Endothelial

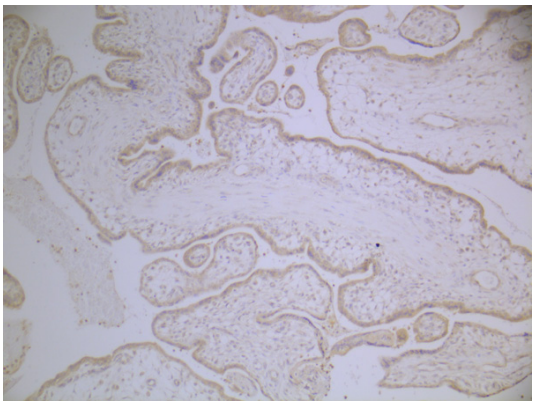

Figure 4. A case with chorioamnionitis stained with prx5. Stromal cells showed more pronounced positivity than in normal cases.

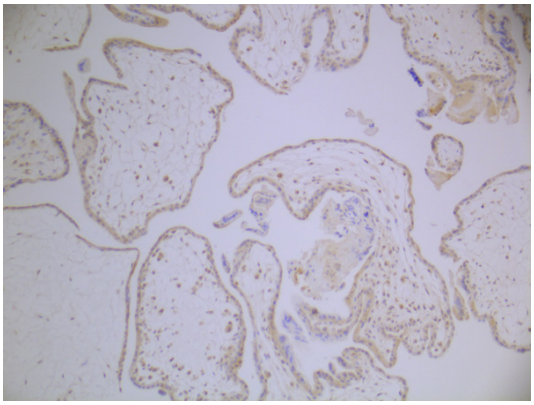

Figure 5. A case of molar disease stained with prx 4. Triphoblastic and stromal cells are positive.

expression of the investigated prxs did not differ during the trimesters. Prx2 expression in different compartments did not associate with the trimester.

Cases with chorioamnionitis had higher stromal prx6 (P=0.048, Fisher's exact test) and prx5 (P=0.042, Fisher's exact test) compared with normal cases (**Figure 4**). Stromal prx6 was also higher in molar disease (P=0.035, Fisher's exact test) and in chromosomal derangements (P=0.013, Fisher's exact test).

Trophoblastic prx4 was more strong in molar disease (P=0.047, Fisher's exact test) than in normal cases (**Figure 5**). There was also a near significant relation for prx5 in molar disease (P=0.052, Fisher's exact test). No other associations were found for trophoblastic expression. For prx6, prx5, prx4 or prx2 endothelial expression did not show any disease specific relation. Endothelial prx3 was, however, associated with chorioamnionitis (P=0.004, Fisher's exact test).

Associations between thioredoxin, 8OHdG and peroxiredoxins

Prx2 associated with epithelial thioredoxin and 8OHdG (P=0.024 and P=0.007, respectively, chi-square).

Stromal thioredoxin associated with all three prxs (P<0.001 for all, chi-square). Trophoblastic thioredoxin expression associated with prx6 (P=0.004, chi-square), prx5 (P<0.001, chi-square) and prx4 (P<0.001, chi-square). Endothelial thioredoxin expression associated with Prx6 (P=0.049, chi-square) and prx5 (P=0.011, chi-square).

Stromal 8OHdG associated with stromal prx6 (P=0.027, chi-square). Trophoblastic 8OHdG expression associated with prx4 (P=0.015, chi-square) and prx5 (P=0.042, chi-square). No significant associations were observed between endothelial 8OHdG and endothelial prx6, prx5 or prx4.

Discussion

In this study we investigated the protein expression of thioredoxin, 8OHdG and peroxiredoxins 2-6 in placental tissues representing all three trimesters and various common diseases such as toxemia, diabetes, hydatidiform mole, chromosomal changes, inflammation and placenta accrete. In earlier studies it has been established that during the vascularization of the placenta ROS burden is increased due to oxygenation and following ischemia reperfusion insult occurring in the end of the first trimester [19]. This is followed by a rise in antioxidative enzymes such as SOD, glutathione peroxidase and catalase [19]. Our results are in line with these observations showing a higher expression of 8OHdG in stromal cells in the first trimester compared to the second and third trimester. This was also the case for stromal trx, prx3, prx 4 and prx 6. We also noticed a higher trophoblastic expression of prx3, prx4 and prx5 during the first trimester.

8OHdG levels did not differ in the endothelial cell compartment of the placental tissues, how-

Table 1. Associations in the reactivity of investigated parameters in different cellular compartments of the placenta

	8OHdG	Trx	Prx2	Prx3	Prx4	Prx5	Prx6	
Stroma	0.011	0.058	0.349	<0.001	<0.001	0.001	0.535	Trophoblast
Stroma	0.026	<0.001	0.149	0.001	0.021	<0.001	<0.001	Endothelium
Trophoblast	0.004	0.184	0.826	0.196	0.420	0.237	0.334	Endothelium

Abbreviations: 8OHdG = 8-hydroxydeguanosine; Trx = Thioredoxin; Prx = Peroxiredoxin.

ever. Vascular invasion of placental villi is regulated by NO which is synthesized by iNOS and eNOS [20]. Since NO is an oxygen containing reactive molecule some of it transforms to peroxynitrite and ultimately to nitrotyrosine in tissues [20]. Nitrotyrosine is found in normal placenta and in pre-eclampsia and diabetes its expression is increased [21]. 8OHdG, on the other hand, is a DNA adduct mainly produced by OH molecules. Hydroxyl ions are formed in the Fenton and Haber-Weiss reactions and they are short lived molecules compared to hydrogen peroxide which also functions as a signaling molecule [1]. The Fenton reaction involves hydrogen peroxide and a metal and the Haber-Weiss superoxide ion and hydrogen peroxide [1]. In excess of hydrogen peroxide the antioxidative capacity of cells to neutralize it may be overwhelmed and hydroxyl ions are formed [1]. Even though short lived, the hydroxyl ions react with nearby molecules and form the 8OHdG adduct with DNA [1]. Considering this it is not surprising that there was an association between 8OHdG levels, thioredoxins and peroxiredoxins at least in stromal and trophoblastic compartments as observed in this investigation. Peroxiredoxins are main neutralizes of H_2O_2. Hydrogen peroxide reacts with the reactive cysteine sites of peroxiredoxins forming an oxidized thiol group [15]. Peroxiredoxins also neutralize peroxynitrite [14]. Oxidized peroxiredoxins are reduced by thioredoxin, thus the association between thioredoxin and peroxiredoxins in placental tissues is reasonable [14, 15]. In case of a high concentration of H_2O_2, peroxiredoxins 1-4, which belong to type 2-Cys group, are hyperoxidised. They can be reduced by sulfiredoxin [22].

The high expression of ROSes and antioxidative enzymes, on the other hand, also produces a heightened signaling activity by hydrogen peroxide during the first trimester influencing the cysteine switch and enzyme reaction associated with them. Hydrogen peroxide induces for instance Ask1 which activates p38MAPK and JNK kinases resulting in apoptosis due to oxidative stress [23, 24]. Apoptosis, however, is low during the first trimester compared with the second and the third ones [25]. Prx and trx, on the other hand, protect cells from apoptosis which may then partly account for a lower apoptotic index observed in the first trimester [26, 27]. However, placental proliferation is stronger during the first trimester [28]. Interestingly, trx stimulates cellular proliferation [29]. Also Prx3 and prx6 are associated with increased proliferation [30, 31].

When analyzing differences in the expression of peroxiredoxin in different diseases of the placenta stromal prx6 and prx5 were significantly higher in chorioamnionitis. Peroxiredoxins are induced in inflammatory situations and there is increased expression of prx1, prx2, prx4, prx5 and prx6 in murine macrophages induced by LPS and IFN-γ which partly depends on TLRs [14]. 8OHdG has been reported to be higher in patients with pregnancy related hypertension [32]. In our cases we did not detect significant difference in the expression of 8OHdG between toxemia and normal placenta. This may partly be due to a lower number of toxemia cases in our material. 8OHdG was lower in cases with detected chromosomal changes. We cannot find any evident reason for this but in exome sequencing of patients with several miscarriages the ALOX5 gene, mutations of which were associated with these pregnancies, affects the oxidative response [33]. However, in molar disease of our cases, 8OHdG levels were higher. In earlier works, a high oxidative stress has been associated with defective placentation and increased oxidative stress has been reported also in molar disease [34].

In molar disease stromal and endothelial trx expression was higher perhaps reflecting the

increased ROS associated with this condition [34]. Curiously, trx expression was lower in blood vessels of placentas of diabetic patients even though ROS is elevated in diabetic placentas [19]. Thioredoxin, however, does not neutralize NO related metabolites such as peroxynitrite which are increased in diabetes [21]. Peroxiredoxins, however, strongly neutralize peroxynitrite but none of them was elevated in diabetic placentas.

There are only few publications of peroxiredoxins in placenta. Prx 3 knockout mice had frequent miscarriages underlining the importance of prx3 in combating ROS during pregnancy [35]. Interestingly, autoantibodies against prx3 and 4 have been suggested to be one factor leading to miscarriages [36]. Prx6 has been shown to be lowered in placentas with intrauterine growth restriction [37]. We did not observe lowered values of prx6 in toxemia in our material, however, stromal prx6 was elevated in chorioamnionitis, chromosomal derangements and molar disease. There is an increase of MnSOD in histological chorioamnionitis compared to non-diseased placenta indicating increased ROS as is generally the case in many inflammations [38, 39]. In molar disease in the same way as with prx6, thioredoxin and 8OHdG were elevated as was also trophoblastic prx4. Evidently impaired placentation and consequent increase in ROS might be due to their upregulation.

When comparing expression of studied parameters in different cellular compartments of the placenta levels of 8OHdG associated in stromal, trophoblastic and endothelial cells suggesting that ROS as assessed by 8OHdG does not vary in different compartment to a significant degree (**Table 1**). There were, however differences in the expression levels of antioxidative enzymes especially between the trophoblastic and endothelial department where none of the antioxidative enzymes showed a significant relation. The reason for this may reflect a different capacity of different cell types to combat oxidative damage or their different quality in doing so.

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Disclosure of conflict of interest

None.

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