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Heat Shock Protein 70 Expression Is Spatially Distributed in Human Placenta and Selectively Upregulated during Labor and Preeclampsia

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Abstract

Placental oxidative stress is a feature of both human labor and the pregnancy syndrome preeclampsia. Heat shock proteins (HSPs) can be induced in cells as a protective mechanism to cope with cellular stress. We hypothesized that HSP 70 would increase during labor and preeclampsia and that expression would vary in different placental zones. Samples were obtained from 12 sites within each placenta: 4 equally spaced apart pieces were sampled from the inner, middle and outer placental regions. Non-labor, labor and preeclampsia were studied. HSP 70 expression was investigated by Western blot analysis. HSP 70 protein expression was increased in the middle compared with the outer area ($p = 0.03$) in non-labor and in both the inner and middle areas compared with the outer area ($p = 0.01$ and $p = 0.02$ respectively) in labor. HSP 70 was increased in the preeclampsia non-labor group compared to the control non-labor group in the inner region ($p = 0.003$) and in the control labor group compared with the preeclampsia labor group at the middle area ($p = 0.001$). In conclusion HSP 70 is expressed in a spatial manner in the placenta. Changes in HSP 70 expression occur during labor and preeclampsia but at different zones within the placenta. The physiological and pathological significance of these remains to be elucidated but the results have important implications for how data obtained from studies in placental disease (and other organs) can be influenced by sampling methods.

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Introduction

The mechanisms that are involved in maintaining a human pregnancy to term, and the switches that lead to a normal labor and pregnancy outcome or indeed an adverse outcome such as miscarriage, preeclampsia, fetal growth restriction or preterm labor, are complex but the role of the placenta is crucial to them all [1–4]. During a healthy pregnancy maternal spiral arteries are dramatically remodeled. They become widely dilated and lose their responsiveness to vasoconstrictive stimuli. Thus blood enters the intervillous space in a non-pulsatile manner and under low pressure [5].

Preeclampsia affects about 2 to 3% of all pregnancies but this can be much higher in underdeveloped countries. It is an important cause of maternal death worldwide and a leading cause of iatrogenic prematurity and fetal growth restriction [6]. In preeclampsia spiral artery remodeling is partial or incomplete [5]. The ensuing high pressure flow results in hydrostatic damage to the placental villi. Furthermore perfusion by intermittent pulses of fully oxygenated arterial blood is thought to lead to fluctuations in oxygen delivery resulting in oxidative stress [4,7]. The maternal syndrome is, at least in part, due to the maternal response to this damaged placenta. This is known as the two-stage model of preeclampsia [7].

Oxidative stress occurs when the production of reactive oxygen species overwhelms the intrinsic anti-oxidant defenses. It may induce a range of cellular responses depending upon the severity of the insult and the compartment in which reactive oxidative species are generated [4,7]. There is irrefutable evidence of placental oxidative stress in preeclampsia, including increased concentrations of protein carbonyls, lipid peroxides, nitrotryosine residues and DNA oxidation [4,8].

Uterine contractions during labor are also associated with intermittent utero-placental perfusion providing the basis for ischemia-reperfusion type injury to the placenta. Doppler ultrasound studies have demonstrated a linear inverse relationship between uterine artery resistance and the intensity of the uterine contractions during labor [9]. Labor is also associated with placental alterations in several pathways linked to oxidative stress [10].

Heat-shock proteins (HSPs) are expressed by all cells and organisms. They have many important physiological functions as well as helping cells to cope with stressful situations. Some HSPs are expressed constitutively while others are induced by a range of damaging insults including heat shock, ischemia, hypoxia, oxidative stress and physical injury [11]. HSPs are named according to their molecular weight. The inducible HSP 70 is one of the best studied HSPs [12].

The aim of this study was to examine the spatial expression of inducible HSP 70 in placentae obtained from women who delivered by cesarean section and were not in labor, by defining precise sampling zones, and then to compare the expression of each zone with the equivalent zone of placentas obtained from women who delivered vaginally following an uncomplicated labor. The second aim was to determine the expression of HSP 70 in normal pregnancy with preeclampsia, both labor and non-labor.

Materials and Methods

Subjects

Human term placentae were collected from pregnant women at the Southern General Hospital, Glasgow. The study was approved by the local ethics committee. Placentae were collected from: (i) women who had uncomplicated pregnancies and delivered at term either vaginally (labor group) or by caesarean section (non-labor group) and (ii) women who had pregnancies complicated by preeclampsia. The number of patients recruited is shown in Table 1. Caesarean sections were performed for obstetric reasons such as breach presentation, previous caesarean section or maternal request. Patient consent was obtained prior to delivery. Preeclampsia was defined as a blood pressure of $>140/90$ mm Hg on at least 2 occasions at least 6 hours apart occurring after 20 weeks' gestation and accompanied by proteinuria (>300 mg/L in a 24 hour urine collection) with no other underlying clinical problems.

Sample Collection

For each patient (6 patients per group), placental samples ($\sim$1 cm^3) were obtained from three sites by taking measurements from the cord insertion point: 0–2 cm (inner position), 2–4 cm (middle position) and 4–6 cm (outer position) of placenta. Within each zone four separate samples were obtained representing the four quadrants (Figure 1). Samples were rinsed and immediately flash frozen in liquid nitrogen. For this study we had performed a power analysis using G*Power 3.1 for Macintosh.

Materials

All chemicals were purchased from Sigma-Aldrich (U.K.) unless stated otherwise.

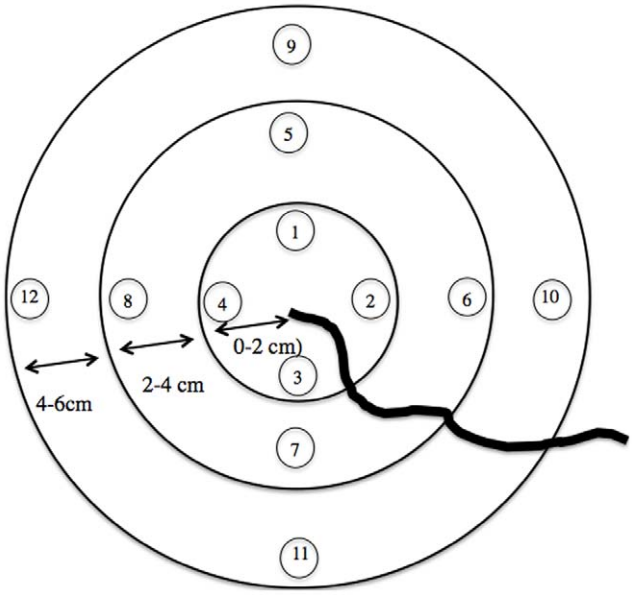

Figure 1. Drawing showing areas where samples were taken from in each individual placenta.
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Tissue Homogenizing for Western Blot

Samples were recovered from storage at -70°C and ground in liquid nitrogen to a fine powder using a mortar and pestle. Tissues was homogenised in the presence of protease inhibitors as described previously [13]. Placenta homogenates were spun at 5000 g for 10 minutes at 4°C to remove debris then supernatants were collected and divided into aliquots and stored at -70°C. Protein concentrations were determined using bovine serum albumin as a standard.

Western Blotting

Western blotting was performed as described previously [13] with some modifications. A volume corresponding to 50 μg of each sample was separated by SDS-PAGE electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide resolving gels. Pre-stained low range molecular weight markers (BioRad) were loaded onto each gel. Transfer of proteins to Hybond ECL nitrocellulose

Table 1. Shows the demographics of patients used for placenta collection.

Category	Normotensive nonlabour n = 6	Normotensive labour n = 6	Pre-eclampsia n = 9	p value
Maternal age (years)	28.33±5.7	26±2.28	31±6.98	ANOVA p = 0.27
Placenta weight (g)	594.7±110.5	589.5±75.0	463.3±139.0	ANOVA p = 0.07
Birth weight (g)	3443±537	3719±347	2545±900*	ANOVA p = 0.01 L v NL (p = 0.32) NL v PE (p = 0.04) L v PE (0.001)
No. primigravid	1	4	6	
Gestation age at delivery (weeks)	39.3±1.0	40.31±1.4	35.86±4.5*	Kruskal Wallis (p = 0.01) L v NL (p = 0.22) NL v PE (p = 0.03) L v PE (p = 0.02)
No. Smokers	2	0	0	

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membranes (Amersham Pharmacia Biotech) was carried out at 22 V and 200 mA for 30 min. Membranes were blocked in 5% donkey serum (Serotec) in TBSTB buffer (20 mM TRIS pH 7.5, 0.5 M NaCl, 0.4% Tween and 0.25% bovine serum albumin) for 1 h at room temperature (RT). Primary antibodies were pre-absorbed in 5% human serum in TBSTB at RT during the blocking process. Membranes were incubated for 1 h at RT with primary antibody solution. The HSP 70 (rabbit polyclonal antibody) was obtained from Enzo Life Sciences (ABI-SPA-812, lot: 09061120) and used at concentration of 1:1000. Membranes were washed and then incubated for 1 h at RT with horseradish peroxidase conjugated donkey anti-rabbit secondary antibody (Abcam (ab7083, lot: gr35152-1) diluted 1:3000 in TBSTB. Membranes were rinsed with TBSTB (2×5 min) and once with distilled water. Filters were re-probed with a β-actin antibody (Sigma) to ensure even protein loading. Immunologically reactive proteins were visualised and quantified as described previously [13]. Statistical analysis was performed using MiniTab on a PC using analysis of variance (Kruskal Wallis for non-parametric data and ANOVA for normally distributed data). Comparison of groups was performed by the Mann Whitney test or student's t-test as appropriate.

Quantitative RT-PCR

Total RNA was isolated using the RNeasy® Midi Kit (Qiagen, 75142). RNA (100 ng) was reverse transcribed into cDNA. Buffers and primers were obtained from the QuantiTect® Kit (Qiagen, 205310) and GoScript™ reverse transcriptase from Promega (A501C). HSP 70 (ID:NCBI 3303) expression (was analyzed by RT-PCR using validated TaqMan® Gene Expression assays with StepOnePlus (Applied Biosystems). b-actin was used as an endogenous control. A positive control human placenta cDNA (Primer design) was used. The relative target gene levels were calculated by comparative CT (ΔΔCT). Statistical analysis was performed as above.

Results

Table 1 shows the demographics of the patients.

Western Blotting

The first set of experiments was designed to test whether there was a difference in HSP 70 expression within individual placentae in both labor or non-labor. Figure 2 shows representative blots of HSP 70 expression in the area sampled 0–2 cm, 2–4 cm and 4–6 cm from the cord insertion point. The upper panel shows a placenta obtained from non-laboring caesarean section delivery. The bottom panel shows a placenta obtained from a women who was in labor and delivered vaginally. Figure 3 shows the results for the mean optical densities for HSP 70 expression for this set of experiments (6 patients in each group). The upper panel shows non-labor and the lower panel shows labor. Overall there was a significant difference between the 3 areas of the placenta for the non-labor group (ANOVA $p = 0.008$). There was significantly more HSP 70 expression in the 2–4 cm (middle) compared with the 4–6 cm (outer) area (student's t-test, $p = 0.03$). No other differences were found (0–2 v 2–4, $p = 0.14$) and (0–2 v 4–6, $p = 0.06$). Overall there was also a significant difference between the 3 areas of the placenta in the labor group (ANOVA $p = 0.002$). There was significantly more HSP 70 expression in both the 0–2 cm and 2–4 cm areas compared with the 4–6 cm areas ($p = 0.01$ and $p = 0.02$ respectively). There was no difference between the 0–2 cm and 2–4 cm areas ($p = 0.3$).

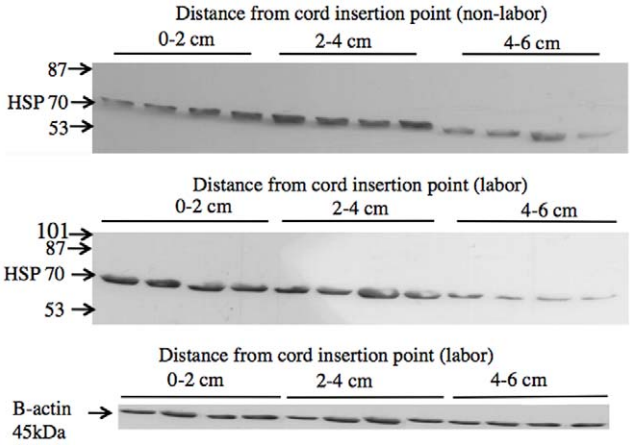

Figure 2. Shows a representative Western blot analysis of HSP 70 expression in placenta of a patient (non-labor) and a patient in labor (n = 6 patients in each group for entire study). Samples are grouped according to distance sampled from cord insertion point. Four samples were obtained within each zone (see Figure 1). Molecular weight markers (kDa) are indicated by arrows. Also shown is a representative β-actin loading control for the gel above showing equal protein loading.
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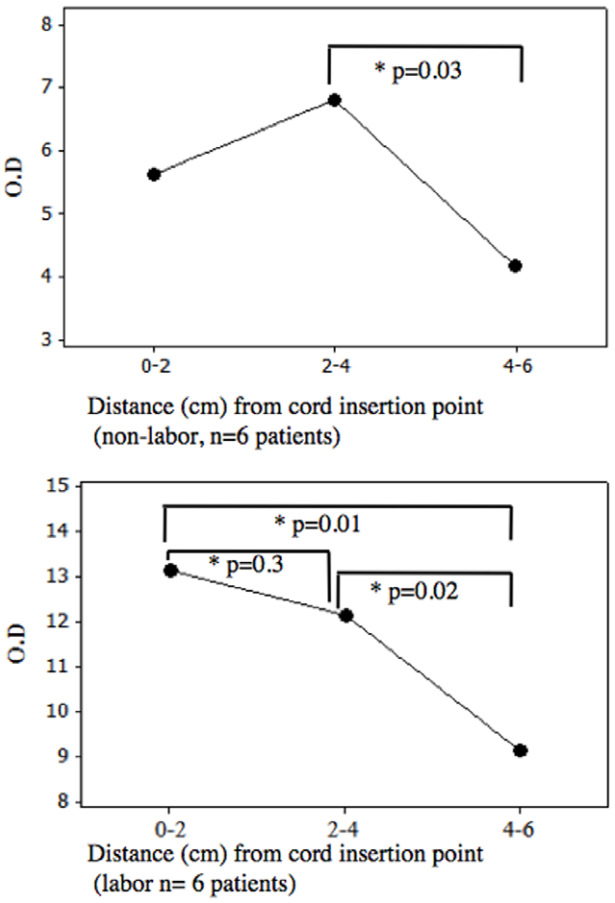

Figure 3. Shows the optical densities for HSP 70 expression in three different placenta zones for all patients. The upper panel shows non-labor (n = 6 patients) and the lower panel shows labor (n = 6 patients).
doi:10.1371/journal.pone.0054540.g003

The second set of experiments was designed to test whether there was a difference in HSP 70 expression between labor and non-labor groups for each of the three sites. Figure 4 shows representative blots of non-labor versus labor for the three different areas of the placenta (upper panel 0–2 cm, middle panel 2–4 cm and lower panel 4–6 cm). Figure 5 shows an interaction plot for HSP 70 showing the relationship between the means of the 3 different areas of the placenta sampled (0–2, 2–4 and 4–6 cm) and the two patient groups (Non-labor solid line (n = 6 patients); labor broken line (n = 6 patients)). Individual groups were then compared using the student's t test. HSP 70 was significantly increased in the labor group when compared to the non-labor group at the 2–4 cm site ($p<0.005$). There was no significant difference in HSP 70 expression between non-labor and labor at the 0–2 cm ($p = 0.99$) or the 4–6 cm ($p = 0.06$) sites.

The third set of experiments was designed to test the difference between HSP70 expression in normotensive pregnancies and pregnancies complicated by preeclampsia. Sample representative blots are shown in Figure 6 for some of the patients. The data is summarised in Table 2. There was a significant increase in HSP 70 expression in the preeclampsia non-labor group (n = 4 patients) compared to the control non-labor group (n = 6) in the 0–2 cm site ($p = 0.003$). This difference was not seen for at the 2–4 cm site. Next the labor groups were compared. There was no significant difference between the control labor (n = 6) and preeclampsia labor groups (n = 5) at the 0–2 cm sites ($p = 0.31$) however there was a significant increase in HSP 70 expression in the control labor group (n = 6) compared with the preeclampsia labor group at the 2–4 cm site (n = 6) ($p = 0.001$).

The next of experiments (Figure 7) was designed to determine if there was any difference in HSP 70 expression in second versus third trimester preeclampsia cases. For all cases combined there were no significant differences noted for either the 0–2 cm sites (median optical density second trimester 24.8), (median optical density third trimester 26) ($p = 0.47$, 95% C.I.) or the 2–4 cm sites (median optical density second trimester 19.9), (median optical density third trimester 19.3) ($p = 0.72$, 95% C.I.).

The final experiment was performed to confirm that the scanning densitometry provided similar results to other quantitative methods. To do this confirmatory experiments were performed as follows. The labour group samples used in experiment one were repeated as above however this time the signals were quantified using the BioRad gel documentation ECL imager system, removing the need for autoradiographs. As for

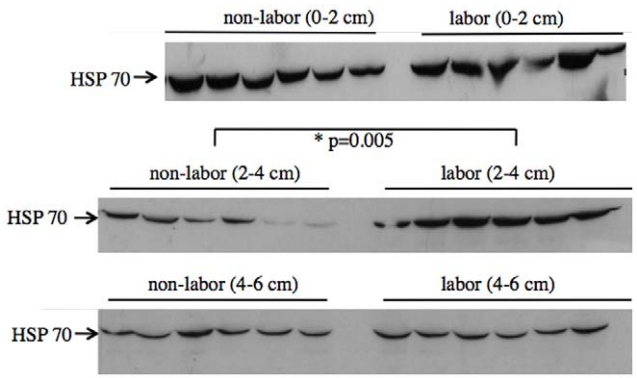

Figure 4. Shows a representative Western blot analysis of HSP 70 expression in labor versus non-labor measured at three distances from the cord insertion point of the placenta: 0–2 cm (top panel), 2–4 cm (middle panel) and 4–6 cm (bottom panel).
doi:10.1371/journal.pone.0054540.g004

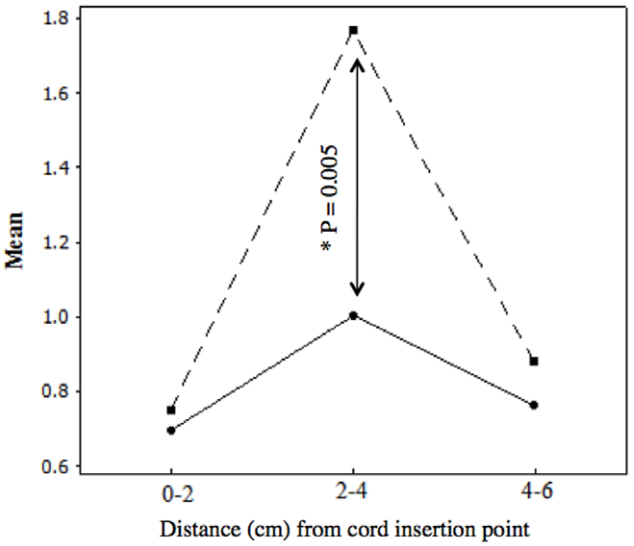

Figure 5. Shows an interaction plot for HSP 70 showing the relationship between the means of the 3 different areas of the placenta sampled (0–2, 2–4 and 4–6 cm) and the two patient groups. Non-labor solid line(n = 6 patients); labor broken line (n = 6 patients).
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Table 2. Shows the median optical density for each group of patients and p value for each comparison from all patients combined for Western blot analysis of HSP 70 expression in non labor control versus non-labor PE at 0–2 cm site, non labor control versus non-labor PE at 2–4 cm site, labor control verus labor PE at 0–2 cm site and labor control versus labor PE at 2–4 cm site.

Group	Group	Sampling site	p value	C.I.
Non Labor group control Median 12.6	Non Labor group PE* Median 20	0–2 cm	0.003	95%
Non Labor group control Median 5.83	Non Labor group PE Median 6.25	2–4 cm	0.41	95%
Labor control Median 12.1	Labor PE Median 16.4	0–2 cm	0.31	95%
Labor control* Median 17.6	Labor PE Median 12.7	2–4 cm	0.001	95%

The representative blot is shown in Figure 6.
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experiment one there was more HSP70 in the inner compared to the outer region and in the middle compared to the outer region (Figure 8 lower panel). A second experiment was performed where a single protein sample was serially diluted (90–10 μg) and HSP70 expression determined. As shown in Figure 8 (upper panel) there was a linear relationship between protein loading and signal intensity which levelled off after 70 μg. This confirmed that the original experiments performed herein (50 μg loaded) were performed with samples within the linear area.

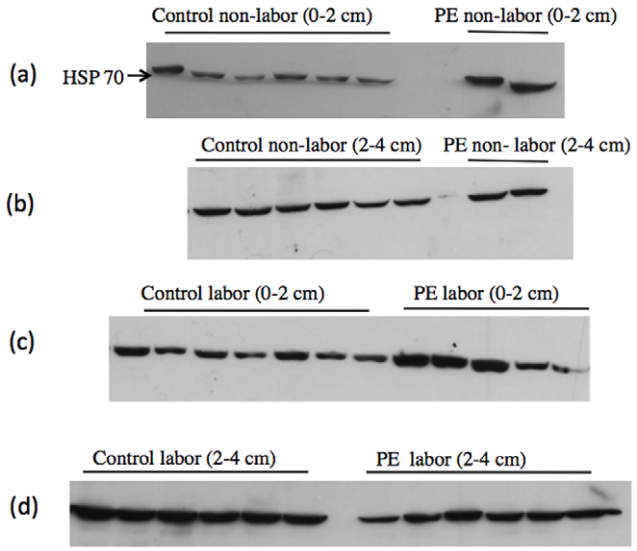

Figure 6. Shows a representative Western blot analysis of HSP 70 expression in labor versus non-labor normotensive and preeclampsia cases measured at 0–2 cm and 2–4 cm from the cord insertion point. Statistical analysis for all gels is shown in Table 2.
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Real Time PCR

There was no differences in any groups except one. The labor control group was increased compared to the labor preeclampsia group ($p = 0.03$) matching the protein findings.

Discussion

This study shows for the first time that HSP 70 is expressed in a spatial manner in the placenta with the highest expression being in the 2–4 cm (middle) area in both labour and non-labour groups. It also shows the importance of using a systematic method to sample the placenta. Most previous reports of placental protein expression do not take this into account. Taking a single or a few samples or averaging protein expression of several samples may well mask possible changes in expression. Apart from the reported changes and their link to placental pathology the results have important implications for how results in placental disease (and perhaps other organs) can be influenced by sampling methods.The increase in HSP 70 in labor and preeclampsia at precise zones suggests that there is a controlled spatial change in HSP 70 expression. The physiological and pathological significance of this remains to be elucidated but oxidative stress is the common link. Oxidative stress occurs when the production of reactive oxygen species overwhelms the intrinsic anti-oxidant defenses.

The main components of the HSP 70 family are HSP 72 (HSP 70i) (induced during cell stress) and HSP 73 (HSC 70) which is constitutively expressed in all cells. Both have very similar amino acid sequences. Both are involved in translocation of proteins from the cytosol into the endoplasmic reticulum and mitochondria and in protein folding during and after synthesis [14,15]. Under non-stressful conditions constitutively expressed members of each HSP family are found in almost all organelles including the nucleus, cytoplasm, endoplasmic reticulum and mitochondria. By interacting with proteins and peptides they play an important role in cell and organ survival. HSPs are induced in response to cell stresses including heat shock, oxidative stress, ultraviolet radiation, ischemia-reperfusion injury, viral infections, nutrient deprivation, hypoxia, physical damage, ischemia and chemicals. Two mechanisms counteract protein misfolding: (i) the molecular chaperones (including HSPs) that facilitate assembly, folding and translocation of proteins as well as the refolding of denatured proteins and (ii) the ubiquitin-proteasome system which regulates the degradation of misfolded proteins which cannot be renatured [16].

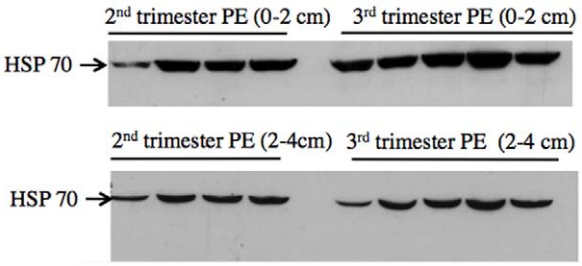

Figure 7. Shows a representative Western blot analysis of placental HSP 70 expression in 2nd trimester preeclampsia cases versus 3rd trimester preeclampsia cases measured at 0–2 cm and 2–4 cm from the cord insertion point.
doi:10.1371/journal.pone.0054540.g007

Although originally thought to bind directly to the signalling receptors TLR2, TLR4, CD40, or CD91 it is now known that HSP 70 binds to scavenging receptors LOX-1, SREC-1, and FEEL-1. On binding to the receptor it is thought that HSP 70 then signals to the TLR2 receptor which in turn signals MyD88 activation leading to the phosphorylation of ERK which can trigger the activation of an undetermined transcription factor that will bind the IL-10 gene promoter leading to IL-10 production [16]. Interestingly IL-10 can be pro-inflammatory at the end of

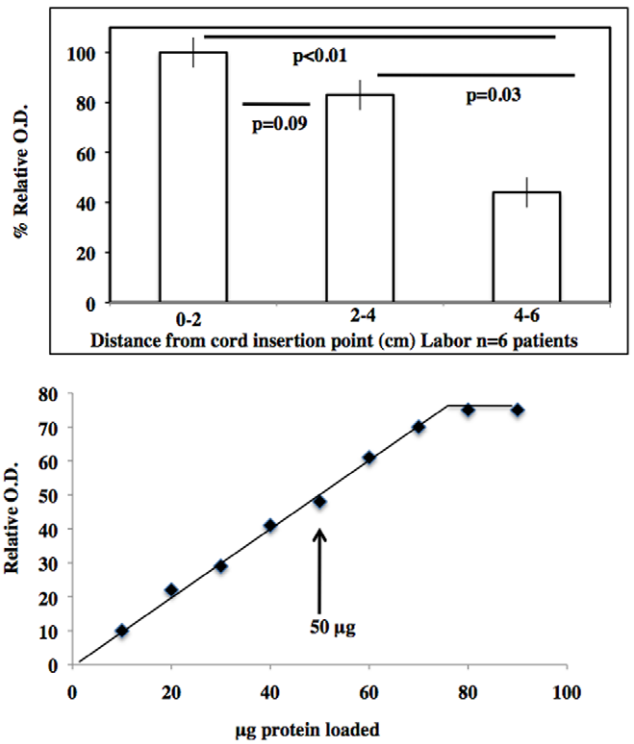

Figure 8. Shows HSP 70 expression in three different placenta zones for all patients in the labor group (n = 6 patients) (upper panel). Quantification was performed using the BioRad documentation ECL imager system. The lower panel shows the relationship between protein loading and signal obtained.
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labor and it has been proposed that this inflammatory action of a usually anti-inflammatory cytokine might accelerate parturition and delivery [17].

Apoptosis has been implicated in both preeclampsia and labor. In the apoptotic pathway, HSPs act at several stages to prevent cell death initiated by stress-induced damage. For example HSP 70 inhibits caspase 3 and 9. Thus it is possible HSP 70 acts to keep the rate of apoptosis in check [14,15,16].

Secreted HSPs, including HSP 70, can take part in immune surveillance. They can capture antigens and interact with receptors on antigen presenting cells. HSP 70 can bind to, and activate, human monocytes, inhibiting the secretion of inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-10 [18].

Previous publications of HSP 70 expression in the placenta and changes during adverse pregnancy have not controlled for sampling and the confounding effects of labor. These studies can be summarized as follows and unless stated otherwise controlling for labor or sampling site was not done.

Shah et al [19] used immunohistochemistry to assess HSP 70 expression in paraffin sections of placentae from normal term pregnancies and reported immunostaining on cell types, both in cytoplasm and nucleus. Site of sampling or labor was not assessed. An immunohistochemical study of HSP 70 expression in pre-term labor, term labor, term non-labor and pre-term cesarean section for preeclampsia or intra uterine growth retardation found no changes in HSP 70 expression on amniochorion and basal plate [20]. The placenta was not examined and controlled sampling was not performed. Increased expression of HSP 70 was reported in placenta of what was termed "placental vascular disease" (preeclampsia, preeclampsia, preeclampsia plus IUGR all combined in one group) compared with term non-diseased placentae [21]. All were delivered by caesarean section. Labor was not studied.

One study reported that HSP 70 was expressed in placenta and reported no difference between labor and non-labor however no data or p values were shown to support this statement and no systematic sampling was performed [22]. Similarly Li et al [21] found no difference between labor and non-labor but similar issues applied. Several years ago we examined HSP70 expression in placentae from normal and preeclampsia with our without IUGR [23]. Others have preformed immunofluorescence on paraffin sections. HSP 70 expression was reported to be increased in preeclampsia [24].

The presence of a uterine artery notch in a mixed group of normal pregnant, preeclampsia and preeclampsia plus IUGR was associated with increased eNOS and HSP 70 in basal plate samples taken from patients who underwent caesarean section. Placental villous tissue was not studied [25].

Some studies have examined HSP 70 expression in early pregnancy. HSP 70 temporarily increases during 8–9 weeks of gestation when blood flow to the placenta is initiated leading to an oxidative stress insult [26]. HSP 70 immunostaining also increased in early pregnancy miscarriage [27]. Janiaux et al [28] examined HSP 70 and nitrotyrosine expression in placentae obtained from surgically terminated pregnancies between 8–13 weeks of gestation. They sampled the inner and outer third. Immunoreactivity for HSP 70 and nitrotyrosine residues was greater in samples from peripheral than from central regions of normal placentas and from missed miscarriages compared to controls. They proposed that oxidative damage to the trophoblast, induced by premature onset of the maternal placental circulation is a key factor in early pregnancy loss.

HSP 70 was reported to be reduced in purified cytotrophoblast cells from preeclampsia cases compared to controls however labor and site of sampling was not studied. The shock of enzyme digestion and cell purification are also confounding factors [29].

Since intracellular HSP 70 binds to the progesterone receptor and functions as a co-repressor of this receptor [30] this may in part explain our results providing a mechanism linking HSP 70 to labor.

HSF-1 is the stress responsive transcriptional activator responsible for the inducible transcription of genes encoding HSPs [31]. Padmini et al [32] reported increased HSP 70 and HSF-1 in placentae from preeclampsia cases compare with uncomplicated pregnancies.

Malyshev et al (1995) [33] showed that oxidative stress increases NFκB which in turn activates nitric oxide synthase, nitric oxide release and subsequently HSP 70 induction in several organs. Blocking nitric oxide synthase activity inhibited HSP 70 induction. We have previously shown that villous eNOS [34], peroxynitrite production [35] and lipid peroxidation [23] are increased in preeclampsia.

HSPs can be detected in the circulation. The few reported studies of HSP 70 serum concentrations in preeclampsia and labor are conflicting [30,36].

In summary spatial changes in HSP 70 expression occur during labor and preeclampsia. The physiological and pathological significance of this remains to be elucidated.

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Author Contributions

Conceived and designed the experiments: FL AA KH. Performed the experiments: AA. Analyzed the data: FL AA. Contributed reagents/materials/analysis tools: AA FL. Wrote the paper: FL AA.

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