Heat Shock Protein 27 Is Spatially Distributed in the Human Placenta and Decreased during Labor

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Abstract

Placental oxidative stress is a feature of human labor. Heat shock proteins (HSPs) play a key role in cellular stress. We hypothesized that placental expression of the small HSP 27 would be altered during labor and expression would vary in different regions of the placenta. Six women in labor who delivered vaginally and 6 women not in labor, who were delivered by Cesarean section, were recruited. Four equally spaced pieces were sampled from the inner, middle and outer regions of each placenta (total 12 samples per placenta). HSP 27 expression was investigated by Western blot analysis and RT-PCR. For non-labor, there was less HSP 27 protein in the inner placenta region compared with both the middle region ($p<0.05$) and outer region ($p<0.05$). For labor, there was also less HSP 27 protein in the inner region compared with both the middle ($p<0.02$) and outer region ($p<0.01$). When the 3 regions of the placenta were compared for non-labor versus labor there was less HSP 27 in the labor group at both the inner ($p<0.05$) and middle regions ($p<0.005$) compared to non-labor. Similar to HSP 27 protein, there was less HSP 27 mRNA in the labor group in both the inner region ($p<0.05$) and middle region ($p<0.02$) compared to non-labor. This study suggests that placental HSP 27 may play a role in labor and is spatially controlled. The results have important implications for how data obtained from studies in the placenta 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 changes that lead to a normal 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].

When the production of reactive oxygen species overwhelms the intrinsic anti-oxidant defenses oxidative stress occurs. It can induce a range of cellular responses depending upon how severe the insult is and the cellular compartment in which reactive oxidative species are generated [4,5].

The contractions that occur during labor are associated with intermittent utero-placental perfusion and could lead to an ischemia-reperfusion type injury to the placenta. Indeed Doppler ultrasound studies have demonstrated a linear inverse relationship between uterine artery resistance and the intensity of the uterine contractions during labor [6]. Labor is also associated with placental alterations in several pathways linked to oxidative stress [7].

Heat-shock proteins (HSPs) are a family of proteins expressed by all cells. They have many important physiological functions, one of the most important being to help 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 [8]. HSPs are named according to their molecular weight. HSP 27 belongs to the family of small heat shock proteins (15–30 kDa). In response to stress, changes in expression of HSP 27 occurs and, like many proteins, HSP 27 function can also be regulated by at the post-translational level [9]. The functions of HSP 27 include protein chaperone, control of apoptosis, regulation of cell glutathione levels, inhibition of actin polymerisation as well as protection against heat shock, oxidative stress and mechanical stress [9]. HSP 27 also plays a role in atherosclerosis [10], in regulation of cytokine production from monocytes as well as expression of toll-like receptors [11].

Since HSP 27 plays a role in oxidative stress and inflammation, both features of labor, we hypothesised that HSP 27 expression would alter during labor in the placenta. Thus the aim of this study was to examine the spatial expression of HSP 27 in placentae obtained from women who delivered by cesarean section and were not in labor and secondly to compare the expression of each zone with the equivalent zone of placentas obtained from women who delivered vaginally following an uncomplicated labor.

Materials and Methods

Subjects

Human term placentae were collected from pregnant women at the Southern General Hospital, Glasgow. All ethics protocols were followed as per Declaration of Helsinki. The study was approved by "Yorkhill ethics committee". Signed patient consent was obtained prior to delivery. Patients were handed an information sheet telling them about the study before being handed the consent sheet. The information and consent sheets were also approved by the ethics committee. All signed consent sheets were stored incase of the need for audit. Placentae were collected from: (i) women who had uncomplicated pregnancies and delivered at term either vaginally (labor group, n = 6) or by caesarean section (non-labor group, n = 6). The labor group were all spontaneous labor and were a tight group (labor time minimum 3 hours maximum 8 hours). All placentae were free of infection, confirmed by the pathology report of every placenta. The non-labor were group were all definitely without labor. All were planned Caesarean sections performed for obstetric reasons: breach presentation (2) previous caesarean section (2) or maternal request (2). The groups studied had no underlying maternal conditions such as hypertension, preeclampsia, diabetes or gestational diabetes or any other medical disorders. There was no fetal pathology such as fetal growth restriction. The details of patients recruited are shown in Table 1.

Sample Collection

For each patient (6 patients per group), placental samples (~1 cm^3) were obtained from three sites by taking measurements from the cord insertion point: inner third closest to cord insertion point (inner zone), middle of placenta (middle zone) and outer third of placenta (outer zone) of placenta. Within each zone four separate samples were obtained representing the four quadrants (Figure 1). Placentae had a central cord insertion. 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 and based the numbers on our previous published work [12].

Chemicals

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

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 [12]. 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 by Bradford analysis using bovine serum albumin as a standard.

Western Blotting

Western blotting was performed as described previously [12] 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

Table 1. Demographics of patients used for placenta collection.

Category	Non-labor (n = 6)	Labor (n = 6)	p value
Maternal age (years)	28.33±5.71	26±2.28	>0.05
Placenta weight (g)	594.7±110.5	589.5±75	>0.05
Birth weight (g)	3443±537	3719±347	>0.05
Gestation age at delivery (weeks)	39.3±1.0	40.31±1.4	>0.05
No. primigravid	2	4	>0.05
No. Smokers	2	0	>0.05

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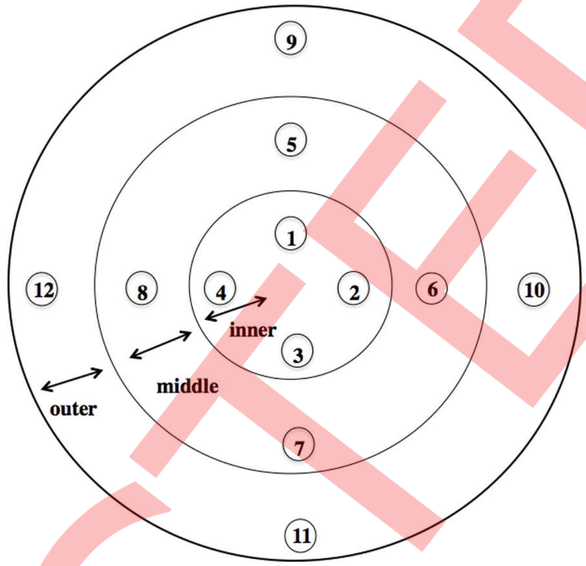

Figure 1. Diagrammatic representation showing where samples were obtained from each individual placenta.
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each gel. Transfer of proteins to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech) was carried out at 22V and 200 mA for 30 min. 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 27 (mouse monoclonal antibody) was obtained from Cell Signalling Technology® (number 2402) and used at concentration of 1:1000. Membranes were washed and then incubated for 1 h at RT with horseradish peroxidase conjugated donkey anti-mouse secondary antibody (Abcam (ab6820) diluted 1:1000 in TBSTB. Membranes were rinsed with TBSTB (2×5 min) and once with distilled water. The same samples were exposed to a β-actin antibody (Sigma) to confirm even protein loading as shown previously [12]. Immunologically reactive proteins were visualised and quantified as described previously [12]. A standard curve was performed for different blot exposures and densitometry was performed when bands were on the linear part of the loading graph as described previously [12]. For each group of experiments the same loading control placenta sample was added to every gel and the densitometry units were normalized to that. We previously confirmed that this method of analysis gives similar findings to other quantitative methods of densitometry [12]. Statistical analysis was performed using MiniTab on a PC using analysis of variance. Comparison of groups was performed by the Mann Whitney test. Graphs show median values along with mean absolute deviation range, a robust measure of the variability of the data.

Quantitative Rt-Pcr

Total RNA was isolated using the RNeasy® Midi Kit (Qiagen, 75142). RNA (100ng) was reverse transcribed into cDNA. Buffers and primers were obtained from the QuantiTect® Kit (Qiagen, 205310) and GoScript^{TM} reverse transcriptase from Promega (A501C). HSP 27 expression was analyzed by RT-PCR using validated TaqMan® Gene Expression assays with StepOnePlus (Applied Biosystems). β-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 C_T ($\Delta\Delta C_T$). Statistical analysis was performed as described 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 27 expression within an individual placenta in either labor or non-labor. Figure 2 shows HSP 27 expression in the four zones of the inner, middle and outer area sampled from the cord insertion point. The upper panel shows a placenta obtained from a 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 combined analysis of all the placentae for either non-labor (upper graph) or labor (lower graph). Overall there was a significant difference between the 3 areas of the placenta for both the non-labor group and labor group (ANOVA $p<0.05$). For the non-labor group there was less HSP 27 in the inner compared with both the middle ($p<0.05$) and outer area ($p<0.05$). For the labor group there was also less HSP 27 in both the inner compared with the middle ($p<0.02$) and outer area ($p<0.01$). Thus HSP 27 is expressed in a spatial manner within the placenta and the distribution patterns are similar in labor and non-labor.

The next set of experiments was designed to test whether there was a difference in HSP 27 expression between labor and non-labor groups for each of the three sites. Figure 4 shows one representative blot of non-labor versus labor for each of the three different areas of the placenta (upper panel; inner, middle panel; middle and lower panel; outer). Each of the 3 blots were performed on separate days so only labor with non-labor can be compared for this. Figure 5 shows the combined analysis for each of the three groups. Overall there was a significant difference between the 3 areas of the placenta when comparing each non-labor group and labor group in each zone (ANOVA $p<0.05$). When individual zones were compared there was less HSP 27 in the labor group at both the inner ($p<0.05$) and middle zones ($p<0.005$).

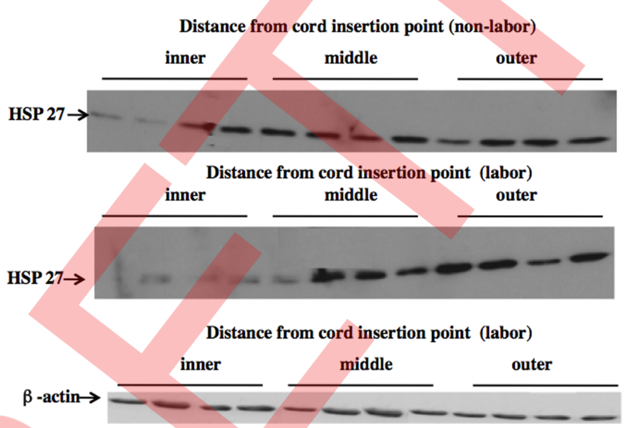

Figure 2. Representative Western blot analysis of HSP 27 expression in the four quadrants of each zone of the placenta in a patient not in labor (upper panel) and a patient in labor (lower panel). (n = 6 patients in each group for entire study). Also shown is a representative β-actin loading control for one set of samples showing equal protein loading.
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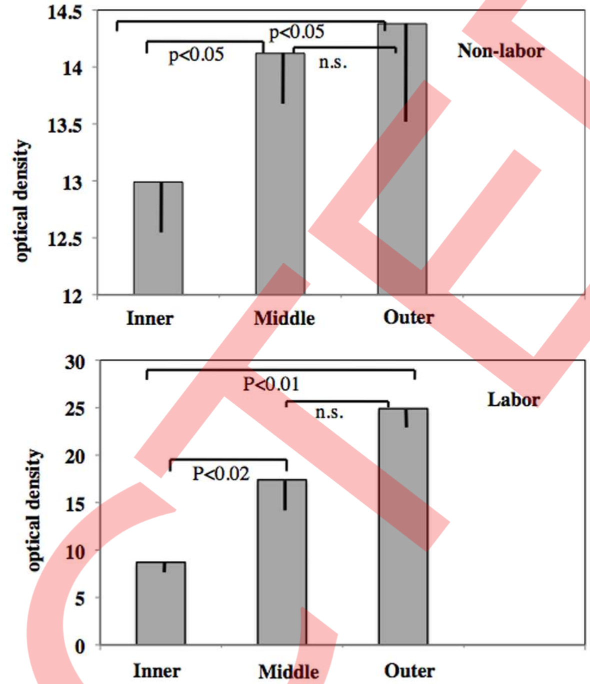

Figure 3. Median optical densities for HSP 27 expression in three different placenta zones for all patients. The upper panel shows non-labor (n = 6 patients) and the lower panel shows labor (n = 6 patients). Samples were compared with the Mann Whitney U test. Values are shown as median and median absolute deviation. n..s., non-significant.
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The final set of experiments was to determine whether the protein changes found in Figure 5 were reflected by changes at the mRNA level. The results are shown in Figure 6. As for HSP 27 protein there was less HSP 27 mRNA in the labor group in both the inner zone ($p<0.05$) and middle zone ($p<0.02$).

Discussion

This study shows for the first time that HSP 27 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

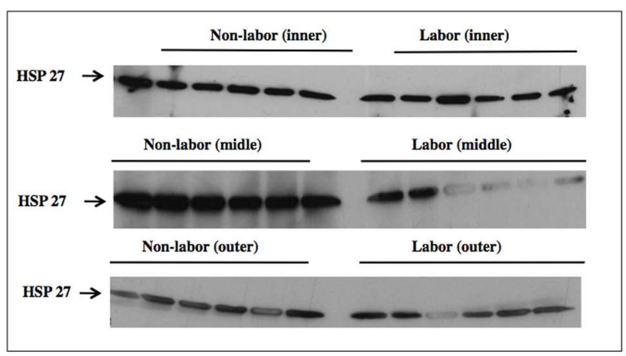

Figure 4. Western blot analysis of HSP 27 expression in non-labor versus labor measured at three distances from the cord insertion point of the placenta: inner zone (top panel), middle zones (middle panel) and outer zone (bottom panel). N = 6 in each group of patients.
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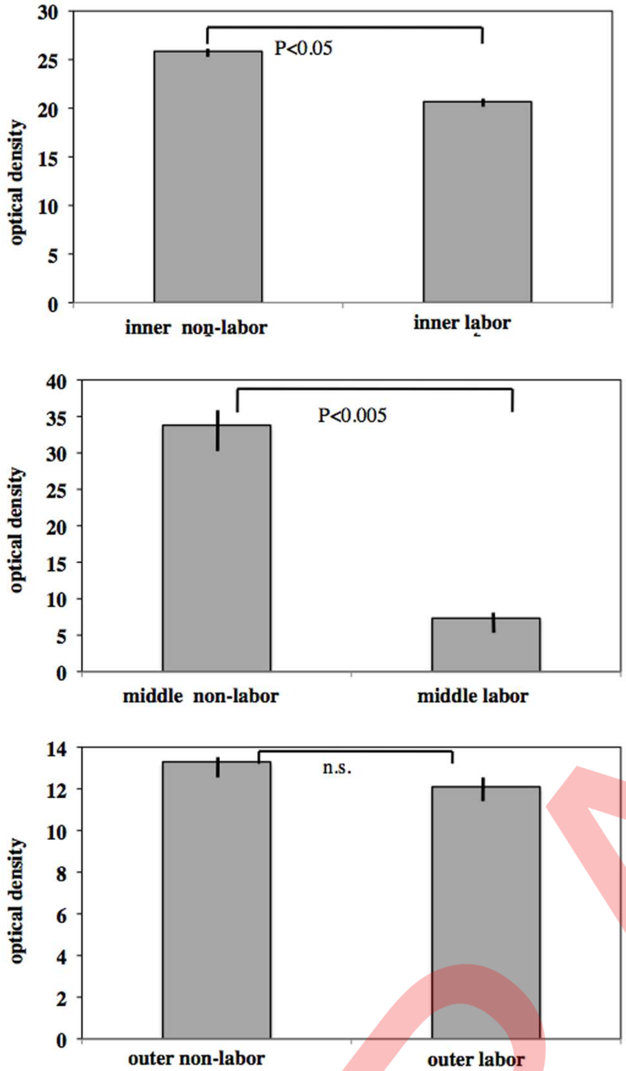

Figure 5. Combined analysis of HSP 27 expression in non-labor versus labor for each of the three zones of the placenta shown in Figure 4. Samples were compared with the Mann Whitney U test. Shown is the median and median absolute deviation. n.s., non-significant.

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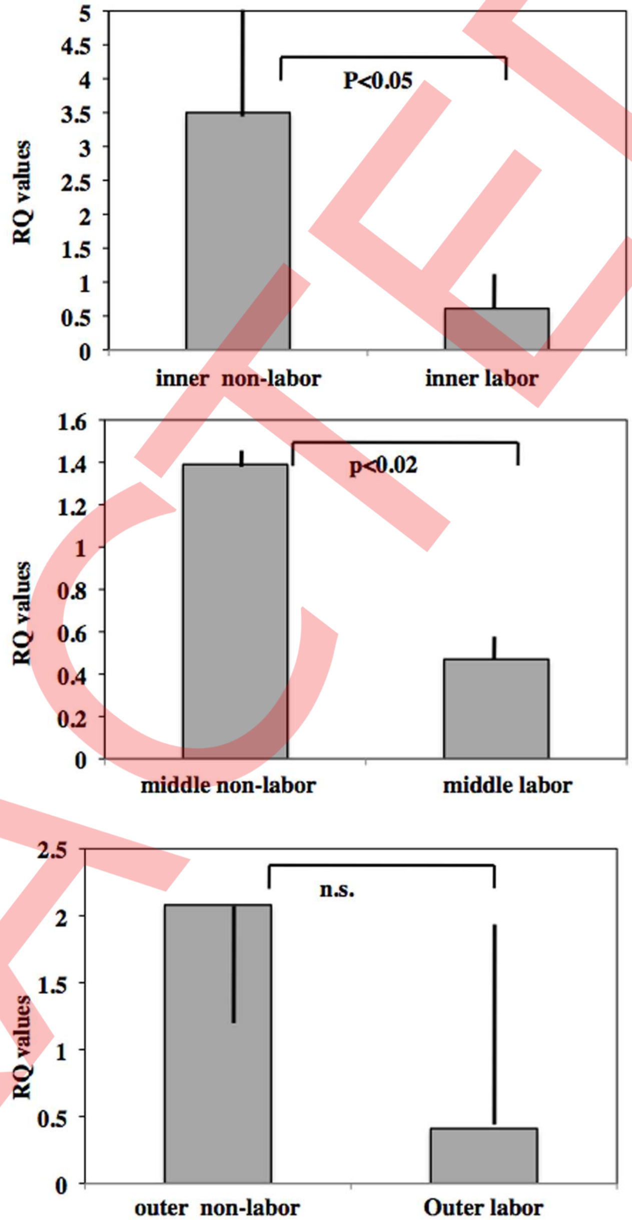

Figure 6. Analysis of HSP 27 mRNA expression in non-labor versus labor for each of the three zones of the placenta. Samples were compared with the Mann Whitney U test. Shown is the median and median absolute deviation. n.s., non-significant.

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therefore 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. The study also shows that HSP 27 protein and mRNA are reduced during labor at defined zones. 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 key finding of this study was the fall in both HSP 27 mRNA and protein at the inner and middle zones of the placenta during labor and which was particularly striking in the middle zone. Many HSPs are increased to protect against stress in disease states [13] however in the present study HSP 27 was reduced. One reason for this may be that a fall in HSP 27 may be necessary to facilitate the inflammatory steps of labor which is, after all, a normal physiological process, not a disease. HSP27 protects against apoptosis, decreases oxidative stress, reduces the pro-inflammatory cytokine balance, stabilizes actin, and inhibits NFκB activation [13] but during labor the opposite effect of these events needs to occur.

Previous publications of HSP 27 expression in the placenta are few. One study examined the expression of HSP 27 in placenta in labor and non-labor [7]. A different approach was taken: since different regions of the placenta were not compared, it is impossible to directly compare the present study with that one.

In another study HSP 27 was reported to be unaltered in the placenta in samples from labor and non-labor. However only one biopsy was taken from each placenta and no quantification analysis was performed or presented [14].

Small HSPs can be modified by phosphorylation. HSP27 can be phosphorylated at serine 15, 78 and 82 by MAPKAPK-2 and 3 [13]. Phosphorylation favours small oligomers to form whereas de-phosphorylation favours formation of large oligomer [13]. Small and large forms may have different functions for example larger forms are important in chaperone and anti-oxidant roles whereas smaller forms are important in actin regulation [13]. The HSP 27 gene contains two functional HSE binding sites, a cAMP response element as well as HSF-1 and 2 binding sites [13]. In view of the findings of this study future studies will be directed to understand whether any changes in phosphorylation of HSP 27 or MAPKAPK-2 or 3 occurs at defined zones during labor.

We have previously examined the expression of HSP 70 in the placenta [12]. In non-labor HSP 70 was reduced in the outer area of the placenta compared with the middle area. In contrast in the present study HSP 27 was reduced in the inner and middle areas compared with the outer area. With regard to labor our previous study showed that HSP 70 was increased in the inner and middle areas, the opposite of the findings for HSP 27. At this stage it is only possible to speculate why such zonal differences exist but may relate to the functions of HSP 27 and 70, some of which differ and some overlap. Placental separation is an important part of labor. Herman et al [15] showed that the process of placental separation from the uterine wall can be divided into three distinct phases i.e latent, contraction/detachment and expulsion. They showed that placental separation is accomplished by means of an orderly multiphasic process with a definite direction and sequence. They found in most cases the placenta separated from the uterine wall in a "down-up" separation i.e initiating from the lower pole. Interestingly cases with a previous Cesarean section had a higher rate of up-down separation. In contrast in the case of "fundal placentae" separation started at the placental poles (bipolar separation) and the central area of the placenta was the last to separate. It would be therefore of interest in a future study to investigate whether there was a link between the zonal distribution of HSP 27 or HSP 70 and the method of placental separation.

Placentas collected at term by cesarean section are not subjected to the stress of labor however one possibility is that zonal differences in HSPs might reflect the fact that labor is not far off and that the molecular steps to allow labor to proceed have started. Thus it would be interesting to compare placentas from the second trimester where labor is not close to determine if such zonal differences still exist. In contrast at labor zonal differences in HSPs may be linked to the response to the stress of labor, extent of exposure to hypoxia or may contribute to the process that allows the placenta to separate at delivery.

Wataba et al [16] showed that HSP 27 and 70 were increased in syncytial knots, avascular villi and the presence of thrombus whereas both were reduced in the presence of infarction suggesting different stresses evoke different responses in HSPs in the placenta and the response may very depending on the area of the placenta exposed to the stress. It has been shown that HSP 27 regulates apoptosis through key components of the apoptotic signalling pathway, in particular, those involved in caspase activation and apoptosis [17]. HSP 70 can also inhibit caspase 3 and 9 [18]. HSP 70, via the TLR-2 receptor, can increase IL-10 production; IL-10 can be pro-inflammatory at labor which may accelerate parturition [19]. This may also explain why HSP 70 increases at labor when HSP 27 decreases. Of interest is the observation that reduced matrix metalloproteinase 2 activity has been shown to be linked to reduced HSP 27 [20]. Whether this is linked to the zonal distribution requires further investigation. The expression of HSP 27 was also reported to be reduced in placentae from SGA neonates although zonal distribution was not investigated [21].

Small HSPs have been studied in myometrium during labor. The myometrium undergoes substantial remodeling at the time of labor including rearrangement of the cellular contractile machinery. Since HSP 27 can modulate actin polymerisation one study investigated changes in small HSPs in the myometrium at labor [22]. A 69% decrease in the small HSP αB-crystallin was found in the myometrium at labor plus multiple isoforms of HSP 27. Immunoblotting using phosphospecific HSP 27 antibodies (HSP 27-serine15, −78, and −82) detected marked changes in HSP 27 phosphorylation at labor. HSP 27-Ser15 was 3.0-fold higher in laboring myometrium. In contrast, levels of HSP 27-Ser82 were 85% less in laboring myometrium. There was no significant change in HSP 27-Ser78. It was proposed that decreased expression of αB-crystallin at the time of labor liberates HSP 27 enabling it to participate in other cellular events such as cytoskeletal remodeling. Clearly the functions and structure of the myometrium and placenta are different, however since both play a role in labor future work should investigate the expression of αB-crystallin within the placenta during labor. Also that particular study highlights how different changes in HSP 27 can occur depending on the cellular event to be targeted. In summary HSP 27 is expressed in a spatial manner in the human placenta and changes in expression occur during labor suggest that HSP 27 may be part of the signaling process of labor and thus warrants further investigation particularly with regard to a role on pre-term labor.

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Author Contributions

Conceived and designed the experiments: FL AA. Performed the experiments: AA AF. Analyzed the data: FL AA. Contributed reagents/materials/analysis tools: FL. Wrote the paper: FL AA. Helped design PCR methods: AF.

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