Title

Functional and Molecular Changes of the Maternal Heart During the Late Pregnancy: A Two-Dimensional Speckle-Tracking Echocardiography Study

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A thesis submitted in partial satisfaction of the requirements for the degree

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by

Shayan Moazeni

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ABSTRACT OF THE THESIS

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Master of Science in Physiological Science

University of California, Los Angeles, 2018

Professor Mansoureh Eghbali, Co-Chair

Professor Arthur P. Arnold, Co-Chair

Abstract

Background: During pregnancy, the hearts of pregnant women undergo several changes that effect its structure and function, which is necessary for the progression of a successful pregnancy. Currently, many publications have shown highly variable reporting in the systolic function pregnant women when compared to non-pregnant (NP) controls. Here we investigated whether two-dimensional speckle tracking echocardiography (STE), a highly sensitive load independent modality that is intended to measure cardiac function, can detect changes in the myocardium in late pregnant (LP) female rats. Moreover, we believe that STE can detect subtle changes that may be attributed to hypertrophy process and/or subclinical myocardial dysfunction.

Methods: Adult female Sprague-Dawley rats were used in NP, LP, and post-partum (PP; day 3, 7, and 14) stages. The hearts and the left ventricles were isolated to measure LV hypertrophy and total RNA was isolated using the Trizol RNA isolation protocol. RNA was then reverse transcribed with a gene-specific primer. Standard western blot analysis was performed using in-vivo whole heart lysates. Transthoracic echocardiography was acquired to monitor cardiac function by conventional parameters (LVEF, FS, and heart rate) and with STE.
Results: As expected LP rats had significantly increased hypertrophy and this was reversed by day 14 after delivery. LP rats showed no statistically significant change in LVEF and FS compared to NP rats. LP rats had significantly reduced global circumferential and radial strain compared to NP rats. Changes were transient as both strain values were reversed by day 14 in the circumferential plane and after 1 day in the radial plane after delivery. Finally, Western Blot analysis showed that phosphorylated Akt/Akt protein levels were decreased ~7-fold at the end of pregnancy compared to NP. Additionally, phosphorylated STAT3/STAT3 was also significantly lower (~4 fold) at the end of pregnancy.
Conclusions: The hearts of LP rats have significantly different strain changes compared to NP when using STE. This study provides reference data on the normal range of maternal cardiac motion with STE and changes in LV systolic function during normal pregnancy. However, whether these changes are an adaptive response or are a response to sub-clinical myocardial dysfunction has yet to be elucidated. In the future, use of STE for early detection of pregnancy-associated cardiovascular complications.

The thesis of Shayan Moazeni is approved.

Soban Umar

Xia Yang

Arthur P. Arnold, Committee Co-Chair

Mansoureh Eghbali, Committee Co-Chair

University of California, Los Angeles

2018

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Introduction

Cardiac adaptations during pregnancy

During pregnancy the hearts of pregnant women and rodents develop a reversible physiological hypertrophy in response to volume overload, mechanical stress, and increased cardiac output (CO) [1]. To date, many mechanisms characterizing the hemodynamic and electrophysiological changes observed during pregnancy have been described, such as increased stroke volume (SV), blood volume, end-diastolic volume (EDV), action potential duration, and prolonged QT intervals [1–5]. Despite the numerous reports on cardiac adaptions during pregnancy, left ventricular (LV) function appears to be a controversial.

Currently, literature in both clinical and pre-clinical studies support that an increased CO is paralleled with a decrease in peripheral vascular resistance (PVR) [4]. However, LV systolic function in healthy pregnant subjects have been variously described as decreased, increased or remained constant when analyzing conventional echocardiographic parameters such as ejection fraction (EF), fractional shortening (FS), and tissue doppler velocity (TDV) [3,6–11]. This discrepancy in the reporting could be a result of differences in heart rate (HR), circulating serum sex steroids, and possibly differences in the stages of pregnancy (1st, 2nd, and 3rd trimester). It is important to note that all of the reported findings on cardiac function during pregnancy use conventional parameters (EF, FS, and TDV) that are load dependent [12–16]. Increased blood volume, venous return, and EDV, all contribute to an increase in preload, which results in an increased SV that is consistent with current literature [1,4,12]. Therefore, the use of these

conventional parameters is limited by the variations in ventricular loading conditions in pregnancy [13].

Pregnancy and Myocardial Strain Imaging

Given the limitations present with conventional echocardiographic parameters, more sensitive and load independent measurements should also be measured to ensure LV function is accurately represented. This is especially important during the late pregnant stage where some investigators have found decreased function and trends of subclinical myocardial dysfunction [10,11,17]. If subclinical myocardial dysfunction is indeed present, they are most likely cardiac maladaptation caused or triggered from the physiological hemodynamic changes during normal pregnancy. While these changes are subclinical at rest, they may become clinically relevant when there is an increased demand on the heart. Therefore, new novel parameters like speckle tracking echocardiography (STE), a type of myocardial strain imaging, can be utilized as it overcomes the limitations that are present in conventional parameters and is a highly sensitive tool that can detect subclinical myocardial dysfunction [12,13,16].

Recently, two-dimensional STE has been shown to decrease significantly in late pregnancy [12]. However, no pre-clinical studies have yet to report normal strain imaging changes in murine models and changes that occur after delivery during the post-partum period. Additionally, no studies have yet to identify if changes in myocardial strain are due to hypertrophy or due to the activity of cardioprotective signaling pathways such as PI3K/Akt/GSK3β and/or MAPK/ERK1/2 that have

been implicated in the heart remodeling process during pregnancy [1,5,6,17–20]. Therefore, we hypothesized two possibilities; 1) The structural changes during pregnancy causes subtle changes in the myocardium that can be detected using STE; and 2) during pregnancy cardiac maladaptation's lead to subclinical myocardial dysfunction that can be detected using STE.

Materials and Methods

Animals

Thirty-one healthy adult (~ 2–3 months old; 250-350 grams) female sprague-dawley rats were used in this study. Rats were distributed into three main groups, non-pregnant (diestrus stage; n=8), late pregnant, (LP, n=8), and post-partum (PP, n=15). All LP echocardiographic measurements and collected organs were completed in the evening of day 20 from gestation. Since the length of gestation in sprague-dawley rats is between 21-22 days, all measurements were conducted on day 20 since our interest in this study is during the late pregnant stage [21]. Additionally, PP designated rats were further placed in different groups to examine if recovery and/or changes are present after 1 (n=3), 7 (n=6), and 14 (n=6) days after delivery. These timepoints were chosen because pregnancy-induced hypertrophy in mice has been shown to be reversed by day 7 after delivery in our previous work [19]. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH Publication No. 85-23, revised 1996). The animal protocol was approved by

the University of California Los Angeles School of Medicine Animal Research Committee.

Cardiac hypertrophy measurements

The hearts of NP, LP, and PP rats were dissected to determine heart hypertrophy and then frozen for molecular analysis. The body weight (BW, g) and total ventricular weight (VW) of the rats were recorded. Additionally, the LV was isolated to measure LV hypertrophy (LV/VW). (Note: normally the LV mass would be normalized with BW to identify LV hypertrophy. However, because of the presence of pubs in the LP rats, this measurement would not be representative of the overall LV hypertrophy).

Echocardiography and strain analysis

Echocardiography was performed to monitor cardiac hemodynamics. Rats were anesthetized with inhaled isoflurane (3% for induction and 2% for maintenance) for echocardiography and HR was normalized amongst all animals to minimize loading conditions that may affect conventional parameters. B-mode and M-mode echocardiography was performed (Visual Sonics Vevo 2100, 25-MHz linear transducer) to evaluate cardiac function. M-mode echocardiography was used to measure LVEF (%), FS (%), and HR (bpm). B-mode images were used in conjunction with Vevostrain, a software designed for STE, to calculate strain changes and are represented as global circumferential strain (GCS, %) and global radial strain (GRS, %). All myocardial strain calculations were obtained with STE

and was measured in triplicates at end-systole. Hearts were removed rapidly under deep anesthesia for preservation of protein and mRNA integrity.

Real-time qPCR

Cardiac tissue was frozen immediately in liquid nitrogen and stored at -80°C to maintain RNA integrity. Total RNA from the heart was isolated using the Trizol RNA isolation protocol and was reversed transcribed with a gene specific primers. U6 was used as used as an internal reference or “housekeeping” gene.

Western blot analysis

Cardiac tissue was frozen immediately in liquid nitrogen and stored at -80°C to maintain protein integrity. Standard western blot analysis was performed using *in-vivo* whole heart lysates from all three main groups.

Statistical analysis

One-way ANOVA was used to assess for statistical significance. When significant overall differences were detected by the one-way ANOVA, pairwise post-hoc comparisons were carried out between groups using the Bonferroni correction to adequately control our overall type 1 error rate at 0.05. A *p*-value of less than 0.05 was considered statistically significant. Values are presented as mean +/- SEM.

Results

Development of heart hypertrophy in late pregnant rats

We assessed myocardial hypertrophy in all three groups (NP, LP, PP). As expected and previously reported in the literature, pregnancy was associated with significant cardiac hypertrophy. The hearts of LP rats had significant hypertrophy evident by an increase in VW (NP: 0.77+/-0.01 g *vs*. LP: 0.89+/-0.03 g *vs*. PPD14: 0.77+/-0.01 g; P=0.05 normalized to NP) and by LV/VW (NP: 0.48+/-0.02 g *vs* LP: 0.57+/-0.03 g *vs* PPD14: 0.49+/-0.026 g; P<0.01, normalized to NP) when compared to NP. Heart hypertrophy was sustained until day 14 after delivery (Figure 1 A and B).

Late pregnancy is not associated with any change in ejection fraction and fractional shortening

LP rats showed no statistically significant change in LVEF (NP: 71.35+/-1.52% *vs.* LP: 67.65+/-1.41%; P=0.10, normalized to NP) and FS (NP: 42.643+/-1.76% *vs.* LP: 39.07+/-0.90%; P=0.09, normalized to NP) compared to NP rats (Figure 2 A and B). Additionally, HR was normalized across all three groups and as such no statistical difference was detected between LP, NP, and PP rats were found (NP: 333.75+/-4.78 bpm *vs.* LP: 338.50+/-16.36 bpm *vs.* PPD14: 331+/-6.23 bpm; P=0.414, normalized to NP) (Figure 3C).

Late pregnancy is associated with a decrease in global circumferential and radial strain

LP rats had significantly reduced GSC (LP: -27.50+/-1.41%; NP: -34.375+/-1.95%; PPD14: -33.725+/-1.73%; P<0.01, normalized to NP) and GRS (LP: 42.77+/-3.41% *vs* NP: 58.3+/-4.97% *vs* PPD1: 52.11+/-7.54%; P<0.01, normalized to NP) when compared to NP rats. The changes in strain are transient as both strain values reversed by day 14 in the circumferential plane and after 1 day in the radial plane after delivery (Figure 3 A and B).

Molecular changes during pregnancy

Western Blot analysis showed that phosphorylated Akt/Akt protein levels in the LP group is decreased by ~7 fold compared to NP (phospho-AKT/AKT LP: 0.13+/-0.1 *vs* NP: 1.0+/-0.14 in NP) (Figure 4A). Phosphorylated STAT3/STAT3 was also significantly lower (~4 fold) at in the LP group (phospho-STAT3/STAT3 LP: 1.48+/-1 vs NP: 4.02±1.73) (Figure 4B). Additionally, using qPCR we identified that LP rats had ~2 fold decrease in Tumor Necrosis Factor Receptor two (TNFRII) (Figure 4C).

Discussion

As the main findings of this study, we report that the changes during late pregnancy is associated with 1) a transient decrease in myocardial strain with no significant changes in conventional parameters; and 2) decreased in phosphorylation and expression in cardioprotective signaling molecules, such as Akt, STAT3, and TNFRII (PI3K/Akt/GSK3β and STAT3/MAPK/ERK1/2 signaling pathway). Based on these results, we speculate that these changes during late pregnant may have cardiac specific maladaptation's that are subclinical at rest and can be detected with STE. While these changes may not be detrimental initially, they may become clinically relevant when there is an increased demand on the heart. Additionally, preexisting cardiovascular complications may also be exacerbated during this period and can be an important indicator for cardiac function if EF is preserved during the peripartum period.

Reduced myocardial strain in late pregnancy

In this study, we observed a transient reduction in GCS and GRS with minimal changes in conventional parameters such as LVEF and FS. This suggests that myocardial strain imaging is more sensitive to changes in cardiac function caused by pregnancy. This result actually is to be expected, since STE directly measures the myocardium as opposed to the fraction of outbound blood (LVEF), which indirectly evaluates myocardial function [13,14].

In this study, we also normalized the HR to minimize loading conditions and their effect on conventional parameters. However, even with the adjustments to HR, the

conventional parameters were still not sensitive enough to be statistically significant [22,23]. We assume that if HR is not normalized, we would observe more variation in the LVEF and FS. Moreover, while LVEF has been variously reported in both clinical and preclinical settings, our results match the results reported in humans [12]. This consistency in the reporting and the low interobserver variability we observed makes us believe that myocardial strain imaging's ability to assess cardiac function is accurate and highly reproducible when utilized by trained technicians, students, and physicians.

Structural and molecular changes in late pregnancy

Based on our hypotheses, myocardial strain imaging was able to detect transient changes in late pregnancy. However, these results could be from a number of changes during pregnancy, like physiological hypertrophy and/or because of subclinical myocardial dysfunction that reduce LV systolic function. While both are possible, and may not be mutually exclusive from one another, further research is needed to elucidate why myocardial strain changes occur.

With the collected frozen heart samples, we identified some subclinical molecular changes during late pregnancy, like decreased Akt and STAT3 phosphorylation and decreased TNFRII expression. However, it is important to note that while some of these markers have been reported during pregnancy, there also seems to be some proteins signaling cascades that are also variously described in the literature [1,6,18,24]. It is our belief that this discrepancy could be due to changes/fluctuating levels of circulating sex steroid that influence specific protein activity or

phosphorylation throughout the duration of pregnancy. Many publications have defined their LP group on day 16-18, whereas we have decided to analyze them on the evening of day 20 [6,24]. This is significant because circulating serum steroid, like progesterone and estrogen, peaks on day 17-18 and rapidly declines back to homeostatic regulated levels through delivery [6,25,26]. This is extremely fascinating as some of the intracellular signaling molecules such as ERK1/2 and STAT3 in neonatal rat ventricular myocytes (NRVMs) have recently been shown to fluctuate activity in an progesterone and estrogen dependent manner respectively [6,27]. Moreover, these two proteins are involved in the extracellular matrix remodeling process that occurs during the reversible hypertrophy process, implicated in cardioprotection, and have been shown to facilitate the type hypertrophy (concentric vs eccentric hypertrophy) in a wide variety of settings [6,28]. Indicating again that the strain changes could be a result from the hypertrophy process and/or subclinical myocardial dysfunction. This late change in circulating sex steroid could also in part be why we see slight cardiac functional decline in the late pregnant stage and why some investigators have found trends of subclinical myocardial dysfunction [10,17].

Future of speckle tracking echocardiography during pregnancy

Based on these results, we encourage the utilization of STE along with conventional parameters in models of pregnancy for research and with pregnant patients. Currently, STE has yet to be adopted to common clinical routine except for in cases of cardio-oncology where cardiotoxic agent like anthracyclines can

cause subtle but life-threatening functional decline [29–31]. While adoption for STE should be evaluated case-by-case, we believe that utilization of STE along with the conventional parameters will provide more cardiac-specific information during the peripartum period. Additionally, it is our belief that STE has the potential to identify early changes in function that could be a result from pathologic complications associated with pregnancy like ischemic heart disease, preeclampsia, and peripartum cardiomyopathy [16,32,33].

While ischemic heart disease or myocardial infarction in pregnancy is not very common, heart disease during pregnancy is the leading cause of nonobstetric mortality. This, in addition to the increased maternal age and changes in women's lifestyle patterns (stress, smoking, diabetes, hypercholesterolemia, chronic hypertension and alcohol, together with physical inactivity), has increased the incidence of ischemic heart disease in pregnant women older than 18 years of age over the past decade [34–37]. As such, early detection and understanding the maternal heart structure, function, and subclinical changes is of clinical importance. Further translational research is needed to validate if strain changes seen in cardiovascular complications associated with pregnancy (i.e Ischemic heart disease, preeclampsia, and peripartum cardiomyopathy) mirror humans. Then, by characterizing molecular pathways, pharmaceutical agents can be created to target those pathways. STE can then be leveraged to measure cardiac functional recovery immediately and because of its sensitivity we can treat cardiovascular complications at earlier timepoints, which can be an important advancement for the management of cardiology patients that are pregnant.

Conclusion

The hearts of LP rats show signs of significant hypertrophy, reduced myocardial strain, and mild but statistically insignificant reduced conventional two-dimensional echocardiographic conventional parameters (LVEF and FS). During late pregnancy, the hearts also showed reduced activity in proteins implicated in cardioprotection. This may explain, in part, why we see reduced function at this time point. The hypertrophy, changes in strain, and LV systolic function all appear to be transient as all groups rapidly reversed during the post-partum period.

Figures

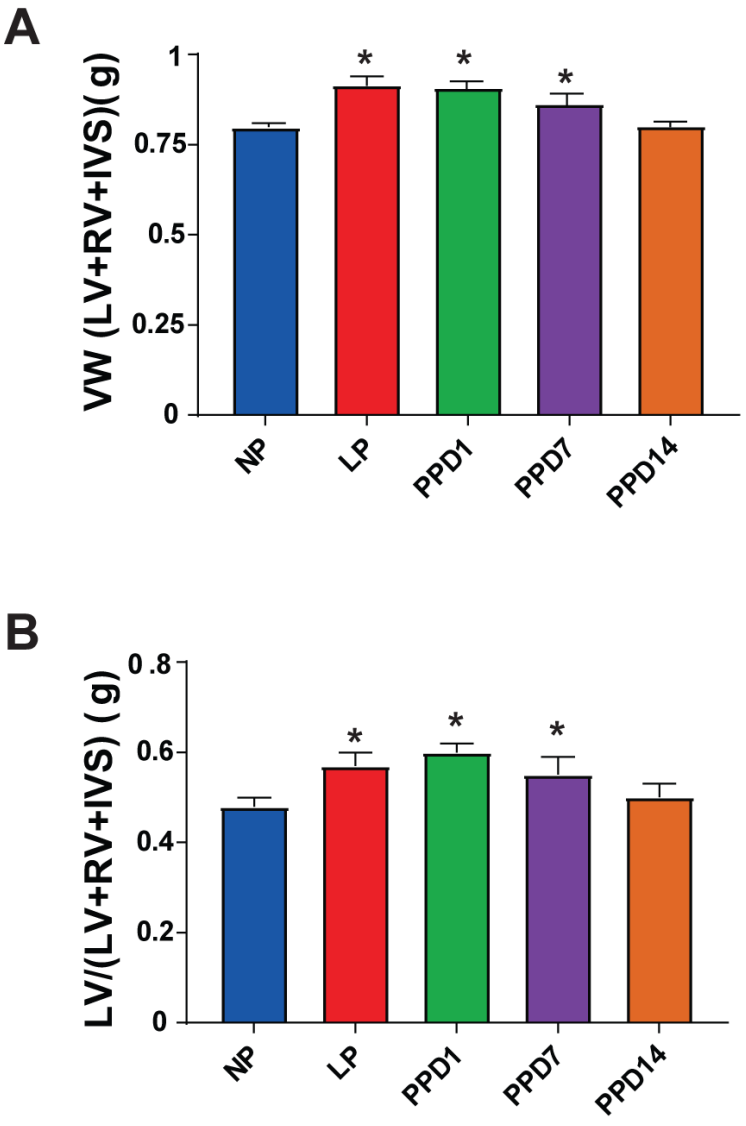

Figure 1: (A) Total ventricular weight measurements indicates that the overall mass of the LP group is significantly larger than NP group (P=0.05 normalized to NP). Recovery was detected 14 days after delivery (B) LP rats showed significant LV hypertrophy (P<0.01, normalized to NP) compared to NP. Recovery was detected 14 days after delivery.

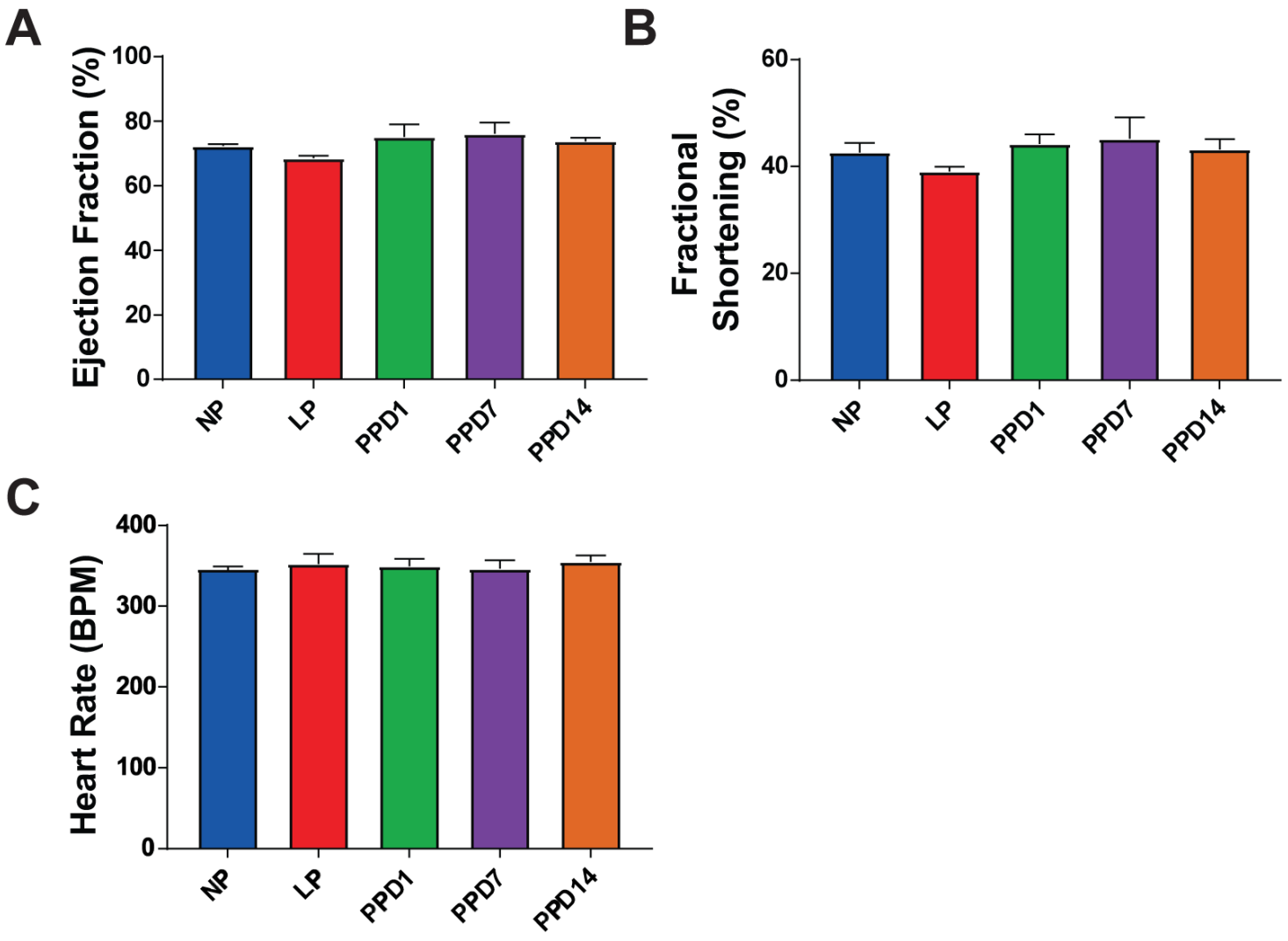

Figure 2: Conventional echocardiographic parameters such as (A) Ejection Fraction (P=0.10, normalized to NP) and (B) Fractional Shortening (P=0.09, normalized to NP) showed no associations with any of the groups (NP, LP, and PP). (C) Heart rate was normalized to minimize loading factors on the heart (P=0.5).

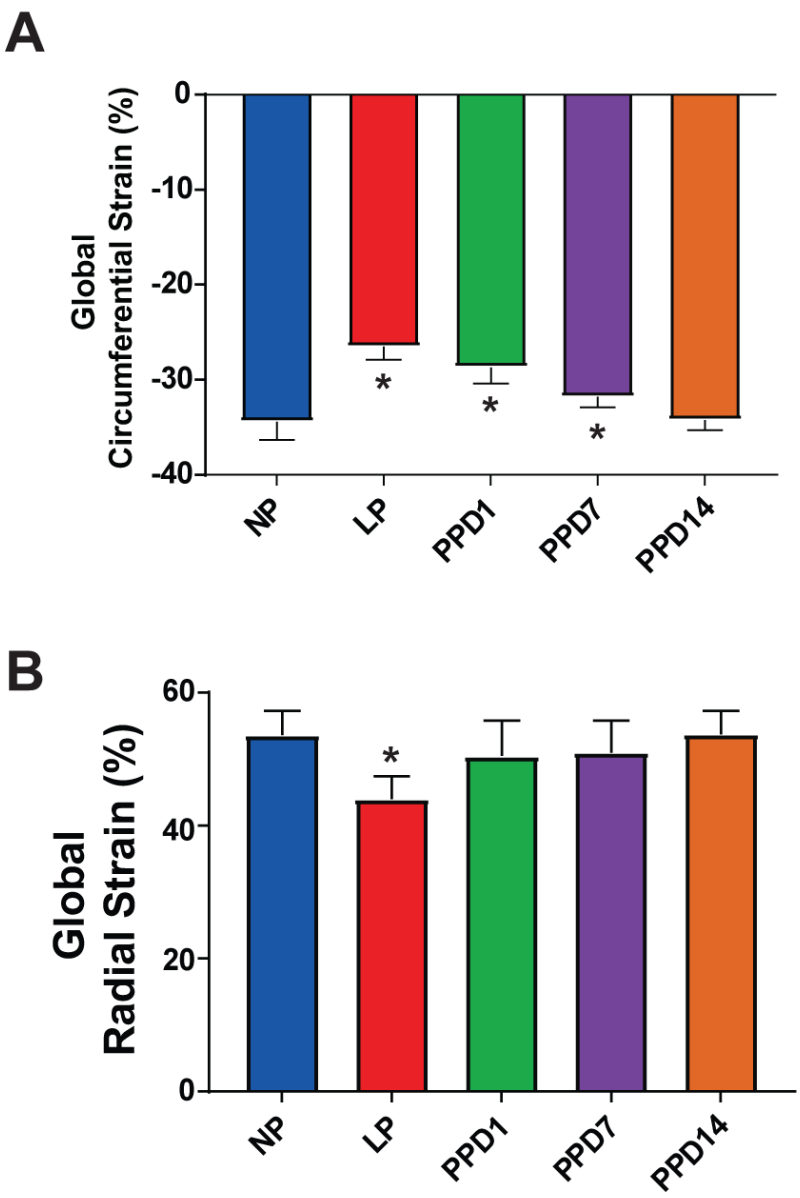

Figure 3: Speckle tracking echocardiographic parameter such as (A) global circumferential strain ($P<0.01$, normalized with NP) and global radial strain ($P<0.01$, normalized with NP) had a transient reduction in strain. However, functional recovery was detected after 14 days after delivery for GSC and only 1 day after delivery for GRS.

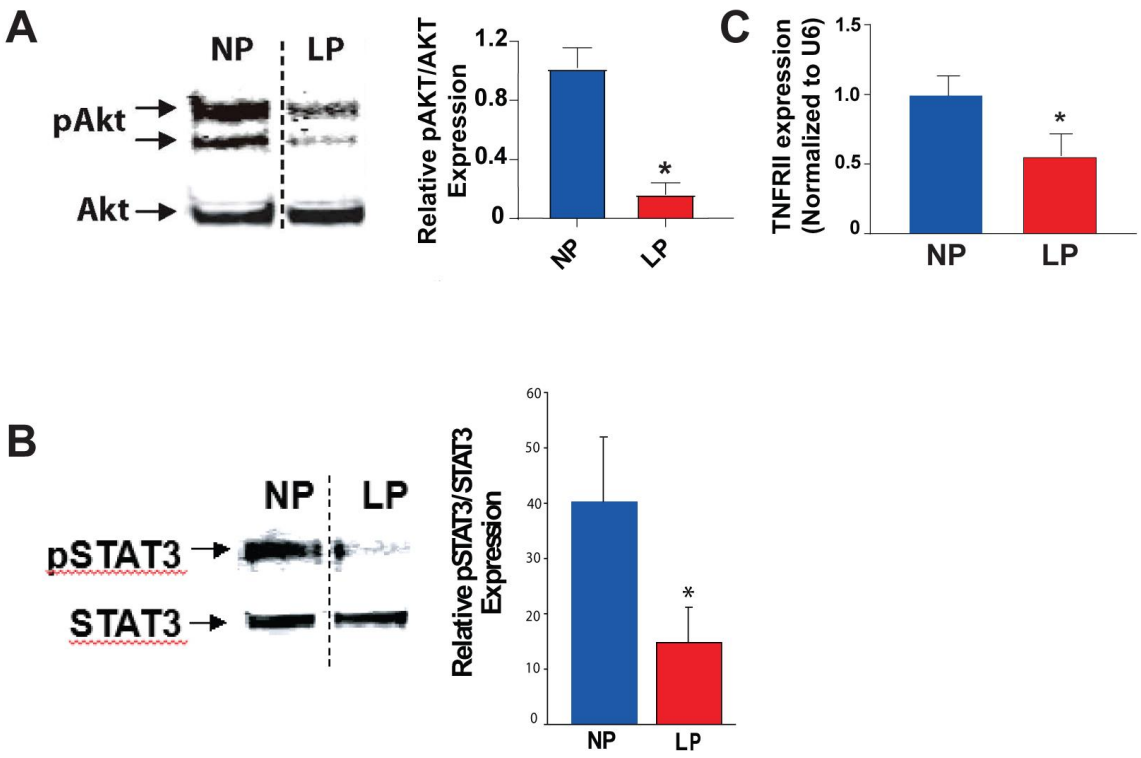

Figure 4: Western blot and qPCR analysis shows a (A) ~ seven-fold decrease in Akt activity ($P<0.05$), (B) ~ four-fold decrease in STAT3 activity ($P<0.05$), and (C) ~ two-fold decrease in TNFRII expression ($P<0.05$).

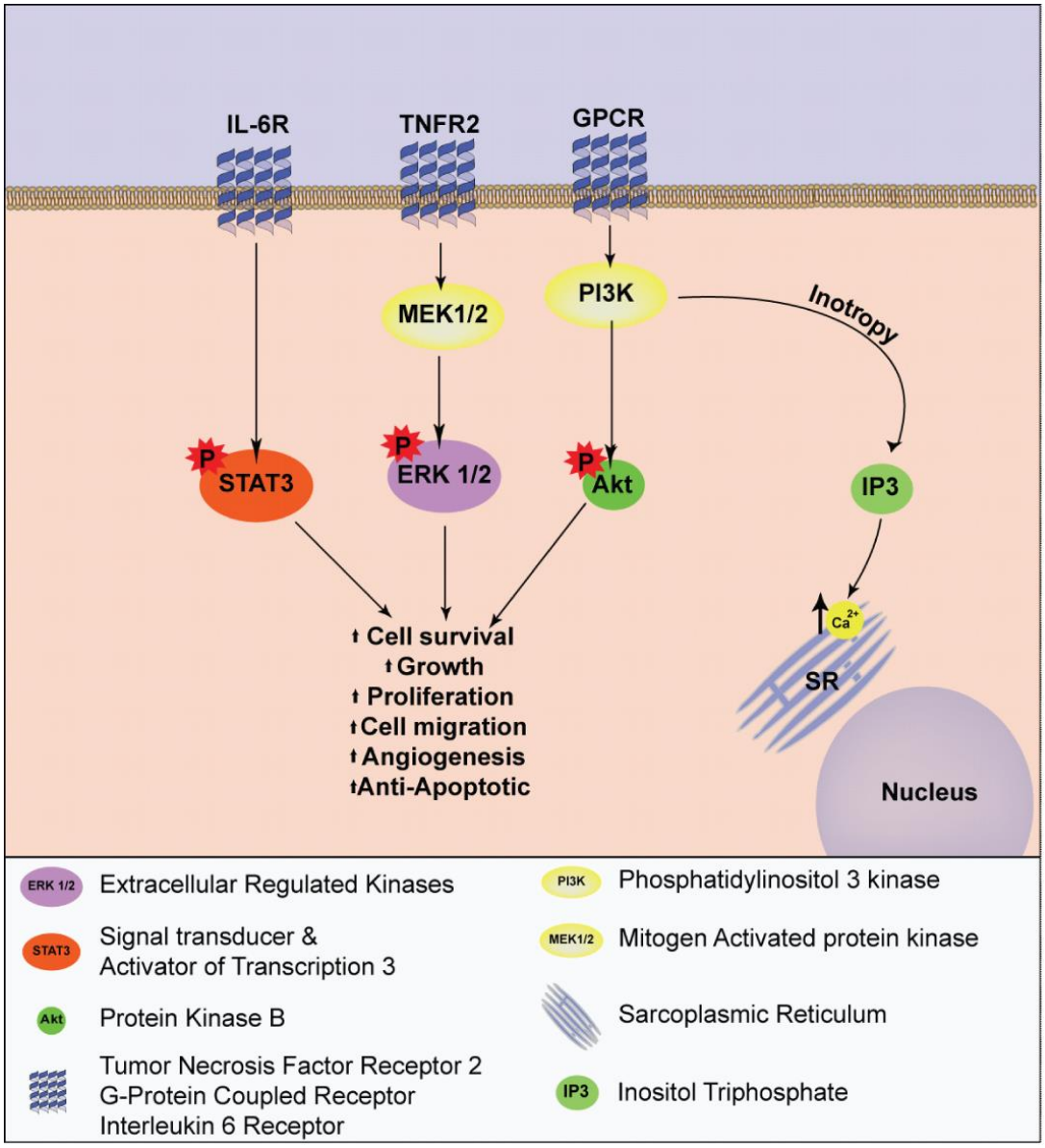

Figure 5: Mechanistic overview of some intracellular signaling molecules play a role in cardioprotection.

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