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RELAÇÃO ENTRE PROTEÍNAS DE CHOQUE TÉRMICO, REPRODUÇÃO MATERNA E DESENVOLVIMENTO FETAL EM DIFERENTES MODELOS DE DIABETE

Felipe Hiroshi Saito

Dissertação de Mestrado apresentado à Faculdade de Medicina de Botucatu – Unesp, Departamento de Ginecologia e Obstetrícia – para obtenção do título de Mestre em Tocoginecologia.

Orientador: Prof. Dr. Steven Sol Witkin
Co-orientadoras: Profa. Dra. Débora Cristina Damasceno
Profa. Dra. Jara Moreno Linhares

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Orientador: Steven Sol Witkin
Coorientador: Débora Cristina Damasceno
Coorientador: Iara Moreno Linhares
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"A Dúvida é o Princípio da Sabedoria"

Aristóteles

Dedicatória

Aos meus pais,

Marilza Taeko Ideriha Saito

Jorge Hisachi Saito

Por sempre me apoiarem em todas as minhas decisões.
Por todo o amor e dedicação.
Serei eternamente grato.
Amo vocês.

"Amor de Família é a coisa mais inexplicável do mundo, nem um pai consegue dizer para um filho o quanto o ama, nem o filho sabe dizer ao pai, então eles simplesmente demonstram ..."

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"Ser profundamente amado por alguém nos dá força; amar alguém profundamente nos dá coragem"

Lao-Tse

"I remember what you wore on the first day
You came into my life and I thought, hey
You know this could be something"

"Não basta ensinar ao homem uma especialidade, porque se tornará assim uma máquina utilizável e não uma personalidade. É necessário que adquira um sentimento, senso prático daquilo que vale a pena ser empreendido, daquilo que é belo, do que é moralmente correto"

Albert Einstein

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Capítulo 1

THE INVOLVMENT OF HEAT SDOCK PROTEINS IN EMBRYONIC AND FETAL DEVELOPMENT

FELIPE HIROSHI SAITO[1], DÉBORA CRISTINA DAMASCENO[1], IARA MORENO LINHARES[2], MARILZA VIEIRA CUNHA RUDGE[1], STEVEN SOL WITKIN[1,3]

[1] Laboratory of Experimental Research on Gynecology and Obstetrics, Department of Gynecology and Obstetrics, Unesp – Univ Estadual Paulista, Botucatu Medical School, Sao Paulo, Brazil

[2] Department of Gynecology, University of Sao Paulo, Medical School and Hospital das Clínicas, Sao Paulo, Brazil

[3] Division of Immunology and Infectious Diseases, Department of Obstetrics and Gynecology, Weill Cornell Medical College, Ney York, New York, USA

*_Correspondence to_: Felipe Hiroshi Saito

Department of Gynecology and Obstetrics

Botucatu Medical School

São Paulo State University, UNESP

Botucatu – São Paulo, BRASIL

Tel./Fax: (+55 14) 3811-6181

Email: pi_saito@hotmail.com

ABSTRACT

Embryo implantation and formation of a functional placenta are essential steps for the establishment of pregnancy, survival and development of the embryo and the fetus in the intrauterine environment. Among the various factors involved in embryo-fetal development, heat shock proteins (hsp) play a central role. These proteins function as molecular chaperones and have a highly conserved amino acid sequence, being essential for survival of all organisms from bacteria to plants and mammals. The hsp are essential for cellular survival, thus it is understandable that hsp have gained considerable interest in almost every medical field including reproduction, immunology and infectious diseases. Recent studies have reported the involvement of hsp in various stages of embryonic and fetal development, from formation of the male and female gametes until parturition. Further studies are needed to elucidate the mechanisms by which hsp influence reproductive processes. The purpose of this review is to present and discuss the involvement of hsp on embryonic and fetal development.

Keywords: heat shock proteins (hsp), fetus, embryo, development, placenta

INTRODUCTION

Embryo implantation and formation of a functional placenta are essential steps in the establishment of pregnancy, survival and development of the embryo and the fetus in the intrauterine environment. Placental dysfunction contributes to an increased frequency of fetal complications (Daskalakis et al. 2008). The processes of fertilization, growth and cell differentiation and the need for the semi-allogeneic fetus to modulate the responses of the maternal immune system require the precise process of developmental events in a clearly defined sequence. Among the various factors involved in embryo-fetal development, heat shock proteins (hsp) appear to play a central role.

Studies have reported the involvement of hsp at various stages of embryonic and fetal development. The heat shock response seems to be an essential component for cellular survival, thus it is understandable that hsp have gained considerable interest in almost every medical field including reproduction, immunology and infectious diseases. Then, researchers have consequently also become focused on development of hsp-based carriers (Mizzen 1998). The purpose of this review is to present and discuss the involvement of hsp on embryonic and fetal development.

HEAT SHOCK PROTEINS

Ritossa (1962) first described the onset of this stress response when *Drosophila melanogaster* were exposed to elevated temperature (heat shock) and gene activity was noted in the form of puffing patterns in their salivary gland polytene chromosomes. The subsequent research led to the identification of the genes expressed differently due to heat stress. These genes were cloned and their protein products called heat shock proteins (Tissières et al. 1974).

Hsp serve at least two major functions. First, under physiological conditions, they act as molecular chaperones (intracellular housekeeping proteins) that are involved in mediating the correct folding and assembly of other intracellular proteins, without altering its final conformation. In addition, they have crucial roles in the prevention of inappropriate protein associations and premature folding, intracellular transport, maintenance of proteins in an inactive form, and protein degradation. Second, their synthesis is greatly up-regulated in response to a wide variety of cellular stresses, such as elevated temperature, and the presence of free oxygen radicals, infections, inflammation, heavy metals, ethanol, and ischemia (Lindquist 1986; Welch 1992). Similarly, under conditions of rapid cellular growth or differentiation such as in early pregnancy heat shock protein synthesis is markedly increased (van Eden 2000; Neuer et al. 1998). Since heat shock protein is a universal cytoprotection protein, it enhances the tolerance to environmental changes or pathogenic conditions, increases the survival rate of stressed cells, and may also play critical roles in cardiovascular disease, organism decay, and cellular aging (Njemini et al. 2007).

Hsp have a highly conserved amino acid sequence and are essential for survival of all organisms from bacteria to plant and mammals (Hunt and Morimoto 1985). These proteins belong to multigene families that range in molecular size from 10 to 150 kDa and are found in all major cellular compartments. In the context of reproduction, the hsp of 60 kDa (hsp60) and 70 kDa (hsp70) are the most important.

Cytosolic molecular chaperones are induced in response to environmental stresses, including hyperthermia, hypoxia, ischemia, inflammation, and oxidative stress (Benjamin and McMillan 1998). During a stressful event, hsp genes are switched on, resulting in increased intracellular protein levels (Child et al. 2006). However, studies have shown that hsp can also be expressed on the cell surface (Multhoff and Hightower 1996; Soltys and Gupta 1997). In vitro physiological stress has also recently been shown to increase levels of extracellular

hsp70 (Guzhova et al. 2001; Hunter-Lavin et al. 2004). It has been suggested that extracellular exposure of hsp will lead to the generation of anti-hsp antibodies (Thomas and Cooper 2002). In addition, hsp60 and hsp70 have been reported to be present in the serum and plasma of healthy nonpregnant individuals, but the source of circulating hsp has not yet been determined (Pockley et al. 1998; Pockley et al. 1999; Lewthwaite et al. 2002). There are evidences that hsp70 may be released from viable cells exposed to stressful insults into the extracellular environment by nonclassical (endoplasmic reticulum-Golgi-independent) protein transport mechanisms: within exosomes or lysosomes, as well as via intact lipid rafts (Broquet et al. 2003; Hunter-Lavin et al. 2004; Lancaster and Febbraio 2005; Mambula and Calderwood 2006). As hsp70 can also be bound to the membrane or embedded in the membrane, microparticles released from cells by membrane shedding need also be taken into account as a potential source of extracellular hsp70 (Molvarec et al. 2010).

Although intracellular hsp70, which are among the most abundant and soluble intracellular molecules (Basu et al. 2000), has anti-inflammatory effects, extracellular hsp70 can act as an intercellular stress signaling molecule, alerting the antigen-presenting cells (APC) and representing an ancestral danger signal to a non-physiological condition, such as cellular stress or damage, whether as a consequence of bacterial and viral infections or mechanical injury, to elicit innate and adaptive pro-inflammatory immune responses (Pockley 2003).

In addition to their intracellular functions, there has been considerable interest in the interaction of hsp with the immune system (Pockley 2003; van Eden et al. 2003). The importance of the interaction of hsp with the immune system is apparent from two important observations: first, the presence of anti-hsp antibodies in serum and, second, cytokine production induced in a number of cell types by exposure to hsp60 or hsp70 (Hunter-Lavin et al. 2004). Hsp of both bacterial and mammalian origin have immuno-modulatory qualities,

both at the level of innate defense mechanisms and at the level of antigen-specific adaptive immunity. At the level of innate defense mechanisms hsp are now known to signal 'danger' to antigen presenting cells such as macrophages and dendritic cells (van Eden et al. 2003). In addition, hsp (hsp70 in particular) may also exert protective effects in the immune system by contributing to the processing and presentation of bacterial and tumor antigens (Jacquier-Sarlin et al. 1994).

HSP IN PRE-IMPLANTATION EMBRYOS

Researchers have reported the involvement of hsp in reproductive processes, beginning at the initial phases of embryo development, formation of the gametes. In the late-1980s, Allen et al. (1988a; 1988b) strongly suggested that hsp70 was present in spermatogenic cells of mice and the developmental regulation of its synthesis indicated that hsp70 might have important functions in germ cell development. Hsp are important, because intrinsic and extrinsic factors can cause DNA damage, interrupt protein synthesis, cell cycle and apoptosis, resulting in abnormal spermatogenesis. Feng et al. (2001) showed that a decreased expression of hsp70 was associated with the pathogenesis of male infertility. These studies support the idea that heat shock proteins play a developmental role during spermatogenesis. Similar to spermatogenesis, hsp expression appears to play an integral role during oogenesis in several animal species, including insects (Ambrosio and Schedl 1984), fish and amphibians (Heikkila et al. 1985; 1997), and also mammals (Heikkila et al. 1986). The conservation of hsp expression in evolutionary diverse organisms supports the assumption of the fundamental role of hsp during germ cell development.

In mice, hsp68 and hsp70 appear during early development (early post-fertilization) and are the main primary products of the zygote (Bensaude et al. 1984). Wittig et al. (1983) showed during the early phases of development (morula or blastocyst stage) when

differentiation of embryonic internal and external cellular mass occurs, the embryo has the ability to express heat shock genes. Therefore, the expressions of hsp seem to be a vital component of the pre-implantation embryo.

In *in vitro* cultured mice (Neuer et al. 1998) and bovine (Matwee et al. 2001) blastocysts, incubation of these embryos in the presence of antibodies to hsp caused a reduction in blastocyst development that may be mediated by an increase in apoptosis. Therefore, the expression of hsp60 and hsp70 on the cellular surface at strategic points seems to be essential for optimal pre-implantation embryo development. Although the mechanisms are unclear, multiple studies reinforce an important role for heat shock proteins in fertilization and early embryonic development (Anderson 1998; Dix et al. 1998; Luft and Dix 1999; Zhong et al. 2011).

HSP IN POST-IMPLANTATION EMBRYOS

During early stages of pregnancy the maternal immune system has to deal with a set of special unique demands: 1) the presence of the semiallogeneic fetus must be tolerated, 2) the growth of trophoblast must be allowed to develop the chorionic villi and ensure a supply of nourishment for the embryo, 3) trophoblast growth must be controlled so that undue invasion is prevented, and 4) the maternal-fetal unit must be protected from infection. Mincheva-Nilsson et al. (1994) showed that, at these stages, the glandular epithelial cells in the maternal decidua express hsp60. A prior sensitization of the maternal immune system to conserved regions of bacterial hsp might lead to the activation of lymphocytes sensitized to human-derived hsp. This could lead to failure in implantation or destruction of embryos in development by several interrelated mechanisms. Hsp recognition by maternal lymphocytes could lead to the induction of pro-inflammatory cytokines that disrupt the balance of immune regulatory mechanisms (Goldsby et al. 2000; Wallin et al. 2002), which help prevent the

rejection of the semi-allogeneic embryo. The rejection could also be a consequence of direct interaction of anti-hsp with hsp expressed by the embryo and/or maternal endometrium.

Mirkes et al. (1999) presented results indicating that hsp70 does play a direct role in the induction of thermotolerance in post-implantation mouse embryos. However, the level of thermotolerance was dependent on the hsp70 level expressed. In addition, the same authors have shown that the hsp27 level is increased when rat embryos are exposed to hyperthermia (Mirkes et al. 1996). The data obtained from post-implantation mammalian embryos support the hypothesis that hsp70 and hsp27 play a role in protecting embryonic cells from the cytotoxic effects of heat and developmental toxicants. The mechanisms by which these hsp protect cells from apoptosis are unknown; however, the known role of hsp as molecular chaperones suggests that hsp may protect cells by interacting with any one of a number of proteins known to regulate apoptosis (Hale et al. 1996).

HSP AND ADVERSE PERINATAL OUTCOMES (APO)

Adverse pregnancy outcomes (APO) are a group of common obstetric diseases, including abortions, intrauterine fetal death, fetal death during parturition, preterm delivery, fetal abnormalities, and intrauterine growth restriction, all of which are far more frequent in the developing world. The most severe adverse outcome of pregnancy is the death of the mother or her offspring (Kramer 2003).

Preterm delivery, defined as delivery before 37 completed gestational weeks, is the major unsolved problem in obstetrics throughout the world. It is the leading cause of perinatal morbidity and mortality. Its short and long-term sequelae constitute a serious problem in terms of mortality, disability, and cost to society (Slattery and Morrison 2002). Approximately 5–10% of all births are premature. Given the heterogeneous etiopathogenesis of preterm delivery, currently, there is no reliable single biomarker with appropriate

sensitivity and specificity for predicting women at risk. According to Yeast and Lu (2007), numerous markers continue to be studied as useful tools in the prevention of preterm delivery.

Fukushima et al. (2005) observed significantly higher serum hsp70 levels in pregnant women at a higher risk for preterm delivery than in healthy pregnant women without any complications. The authors suggested that these elevated levels of hsp70 may function as a marker to predict preterm delivery and evaluate the curative effects of successful intervention. Increased concentrations of hsp70 in mononuclear cells from peripheral blood obtained from women in early pregnancy were associated with subsequent miscarriages, stillbirths and preterm births (Tan et al. 2007). A study by Sotiriou et al. (2004) showed increased expression of hsp70 and hsp90 in chorionic villi of abortions that occurred in the first quarter compared to placentas at term, contributing to the abortion process. Chaiworapongsa et al. (2008) proposed that the mechanisms of preterm and term parturition in humans may involve extracellular hsp70. Furthermore, the presence of anti-hsp60 and anti-hsp70 antibodies in the serum and formation of hsp60- and hsp70-immune complexes in the placenta were also associated with preterm birth (Ziegert et al. 1999). In marked contrast, investigating hsp expression in placental and decidual tissues, Divers et al. (1995) verified that their expression was constant from the third trimester to term and that there was no alteration of these proteins in deliveries at different gestational ages, suggesting that hsp may not be associated with preterm birth.

In the mid-1980s, it was suggested that the response to induction of heat shock in mammalian embryos during the critical period of organogenesis can alter the program of activation and inactivation of genetic loci essential for normal intrauterine development, resulting in anatomical malformations (German 1984). The precise period during gestation when the stress is administered determined the nature of the abnormalities.

A variety of developmental abnormalities have been observed in heat-treated rat embryos (Walsh et al. 1985). Heat exposure resulted in four phenotypes: microphthalmia, microcephaly, gross reduction in forebrain region, and open neural tubes. The severity of the deformity was dose-dependent, with all the defects due to failure of normal ectoderm induction. Interestingly, embryos pretreated with a mild, nonteratogenic exposure to heat to elicit hsp production were protected against a subsequent exposure which otherwise caused severe craniofacial defects.

Child et al. (2006) reported that serum anti-hsp70 antibody levels are significantly elevated at 16 weeks of gestation in women who later gave birth to babies with birth defects (cleft lip or palate or neural tube defects), suggesting a prior increase in hsp70 expression. However, the authors did not find a significant difference in the levels of hsp70 between mothers who gave birth to babies with congenital malformations and to those who gave birth to healthy babies. The authors suggested a possible buffering role for hsp70 in evolution. The maternal physiological stress elicited during the early stages of pregnancy may redirect hsp70 from essential chaperoning duties during fetal development, resulting in birth defects. However, it is still unclear whether hsp are involved directly or indirectly in the pathogenesis of anatomical malformations.

Hnat et al. (2005) found no significant difference in total hsp70 expression in villous tissue from placentas of intrauterine growth restriction (IUGR) pregnancies as compared to normal pregnancies, suggesting that hsp70 does not have a vital role in preeclampsia with or without IUGR. Conversely, the expression of hsp70 at both the mRNA and protein level was up-regulated in placental tissue and microvascular endothelial cells from complicated pregnancies with placental vascular diseases. In addition, the authors found a negative correlation between total hsp70 expression and birth weight. This provided evidence that

hsp70 may play a role in the pathogenesis of that disease (Liu et al. 2008). Further studies are required to clarify the association of circulating hsp70 with intrauterine growth restriction.

HSP AND PLACENTA

The immune system is active at every stage of the reproductive process, from when the sperm fertilizes the oocyte to the birth of the neonate. Immune regulatory mechanisms, some of which are unique to pregnancy, are critically involved in the maintenance of pregnancy (Weetman 1999).

One of the factors involved in this process is the expression of hsp, which increases under conditions of rapid cell growth and differentiation, or following exposure to an environmental stress such as inflammation, fever or toxic chemicals (Ellis 1996). The regions of the decidua and placenta are areas of high metabolic activity and therefore represent potential sites for high levels of hsp expression (Shah et al. 1998). The authors have demonstrated that the hsp90, hsp70 and hsp60 in human placenta were expressed during the third trimester of pregnancy and observed that the intensity of labeling for hsp70 and hsp90 in intermediate trophoblast and decidua decreases with advancing gestation. Wataba et al. (2004) demonstrated the fundamental importance of hsp for cell viability and placental function. These same hsp were expressed in human endometrium during different phases of the menstrual cycle (Ciocca et al. 1996; Tabibzadeh et al. 1996), in the decidua during early pregnancy (Neuer et al. 1998), and also in the umbilical cord (Li et al. 1996).

CONCLUSION

This review has shown that hsp are essential for the formation and development of the embryo during various stages of gestation. Beginning with formation of the gametes, through the pre-implantation stages, and up until parturition, hsp are present and playing important

functions during every stage of pregnancy. However, there are a great number of unanswered questions related to the mechanisms by which hsp act on these reproductive processes. Further studies are therefore needed to elucidate answers to these questions.

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Capítulo 2

HEAT SHOCK PROTEIN PRODUCTION AND IMMUNITY AND ALTERED FETAL DEVELOPMENT IN DIABETIC PREGNANT RATS

FELIPE HIROSHI SAITO[1], DÉBORA CRISTINA DAMASCENO[1], BRUNA DALLAQUA[1], IARA MORENO LINHARES[2], MARILZA VIEIRA CUNHA RUDGE[1], IRACEMA DE MATTOS PARANHOS CALDERON[1], STEVEN SOL WITKIN[1,3]

[1] Laboratory of Experimental Research on Gynecology and Obstetrics, Department of Gynecology and Obstetrics, Unesp – Univ Estadual Paulista, Botucatu Medical School, Sao Paulo, Brazil

[2] Department of Gynecology, University of Sao Paulo, Medical School and Hospital das Clínicas, Sao Paulo, Brazil

[3] Division of Immunology and Infectious Diseases, Department of Obstetrics and Gynecology, Weill Cornell Medical College, Ney York, New York, USA

*_Correspondence to_: Felipe Hiroshi Saito

Department of Gynecology and Obstetrics

Botucatu Medical School

São Paulo State University, UNESP

Botucatu – São Paulo, BRASIL

Tel./Fax: (+55 14) 3811-6181

Email: pi_saito@hotmail.com

ABSTRACT

We evaluated an association between the concentrations of heat shock proteins (hsp60 and hsp70) and their respective antibodies, alterations in maternal reproductive performance and fetal malformations in pregnant rats with hyperglycemia. Mild diabetes (MD) or severe diabetes (SD) was induced in Sprague-Dawley rats prior to mating; non-treated non-diabetic rats (ND) served as controls. On day 21 of pregnancy, maternal blood was analyzed for heat shock proteins and antibodies; and fetuses were weighed and analyzed for congenital malformations. The concentrations of hsp60 and hsp70 were correlated with the level of their antibodies, (R=0.7493, P<0.0001) and (R=0.4789, P=0.0154), respectively. Hsp and anti-hsp levels were correlated with blood glucose levels at several stages of pregnancy. There was a positive correlation between hsp60 and hsp70 levels and the total number of malformations (R=0.5908, P=0.0024; R=0.4877, P=0.0134, respectively) and the number of malformations per fetus (R=0.6103, P=0.0015; R=0.4875, P=0.0134, respectively). The Anti-hsp60 concentration was correlated with the number of malformations per fetus (R=0.3887, P=0.0451) and the anti-hsp70 level correlated with the total number of malformations (R=0.3999, P=0.0387). Moreover, both hsp and anti-hsp showed negative correlations with fetal weight. The results suggest that there is a relationship between hsp60 and hsp70 levels and their respective antibodies and alterations in maternal reproductive performance and impaired fetal development and growth in pregnancies associated with diabetes.

Keywords: diabetes, pregnancy, malformation, heat shock protein

INTRODUCTION

Diabetes mellitus is one of the major chronic diseases and a universal health problem that affects all socioeconomic classes and populations of both developed and developing countries. It decreases the quality of life and promotes a significant burden on health systems (Toscano 2004). *Diabetes mellitus* comprises a group of metabolic diseases characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association 2011). Impaired reproductive performance is a well-known result of the diabetic syndrome in many mammalian species, including humans (Chieri et al. 1969; Kirchick et al. 1978; Vomachka and Johnson 1982; Garris et al. 1986).

Embryo implantation and formation of a functional placenta are essential steps for the establishment of pregnancy and survival of the embryo and fetus in the intrauterine environment. Placental dysfunction contributes to an increased frequency of fetal complications in diabetic pregnancies (Daskalakis et al. 2008). The processes of fertilization, growth and cellular differentiation and the need for the semi-allogeneic fetus to modulate the responses of the maternal immune system require precise process of the developmental events along a clearly defined sequence. Heat shock proteins (hsp) play a crucial role in embryo-fetal development. They have a universal role as molecular chaperones in all species, participating in the folding and unfolding of other proteins, promoting intracellular peptide transport, preventing protein aggregation and denaturation under conditions of stress (van Eden 2000; Neuer et al. 1998).

Hsp are involved in the every stage of the reproductive process from formation of the male and female gametes to fertilization and post-fertilization development (Neuer et al. 2000; Anderson 1998; Dix et al. 1998; Luft and Dix 1999; Zhong et al. 2011). In mice, the 68-70 kDa hsp appear in early development and are the main primary products of the zygote (Morange et al. 1984). The 70 kDa hsp (hsp70) is present at the blastocyst stage during

differentiation of the embryonic internal cellular mass (Wittig et al. 1983). The expression of hsp appears to be a vital component of the pre-implantation embryo. In mice (Neuer et al. 1998) and bovine (Matwee et al. 2001) blastocysts cultured in vitro, introduction of antibodies to the 60 kDa hsp (hsp60) and hsp70 (anti-hsp60 and anti-hsp70, respectively) significantly inhibited further embryo development.

Elevated maternal production of hsp may be indicative of pregnancy-related problems. Serum levels of hsp70 were higher in women who subsequently deliver preterm (Fukushima et al. 2005). Increased concentrations of hsp70 in mononuclear lymphoid cells in early pregnancy were associated with subsequent miscarriages, stillbirths and premature births (Tan et al. 2007). Antibodies to hsp70 in women have also been associated with the presence of congenital defects (Child et al. 2006).

There is increasing evidence that hsp70 is intimately involved with the development of hyperglycemia and *Diabetes mellitus*. Elevated glucose levels are highly associated with reduced systemic hsp70 expression in both humans (Chung et al. 2008) and non-human primates (Kavanagh et al. 2009). A possible mechanism is suggested by the observation that the ingestion of glucose resulted in a decreased ability to induce hsp70 (Febbraio et al. 2004).

In the present study we evaluated whether an association exists between the concentrations of hsp60 and hsp70 and their respective antibodies and alterations in maternal reproductive performance and fetal malformations in association with different models of diabetes (levels of hyperglycemia).

MATERIAL AND METHODS

Induction of diabetes

All experimental procedures were approved by the Ethics Committee on Animal Experiments of the Botucatu Medical School – UNESP (Protocol number 787). Sprague-Dawley female adult rats, maintained under controlled conditions (temperature 22 ± 2 °C, humidity 55 ± 5 % and 12 h light/dark cycle), were randomly assigned to three experimental groups: Non-diabetic (ND, n=5), Mild diabetic (MD, n=12), and Severe diabetic (SD, n=8). For induction of the MD group, newborn female rats received streptozotocin (STZ - Sigma, St. Louis, MO, USA), a beta (β)-cytotoxic agent, diluted in citrate buffer (0.1 M; pH 4.5) at a dose of 100 mg/kg on the first day of life by subcutaneous administrations (Sinzato et al., 2011). For induction of the SD group, rats received streptozotocin diluted in citrate buffer, intravenously at a dose of 40 mg/kg, on day 90 of age (Damasceno et al. 2011). ND rats received only the vehicle (citrate buffer).

Mating

At adult age (day 100), female rats were mated with non-diabetic males. The morning when spermatozoa were present in the vaginal smear was designated day 0 of pregnancy. The mating procedure consisted of 15 consecutive days, which comprised approximately three estral cycles. Non-mated female rats in this period were considered infertile and excluded from the study.

Pregnancy

On days 0, 7, 14 and 20 of pregnancy, in the late evening, blood glucose levels were measured and the mean of blood glucose level calculated. On days 0 and 21 of pregnancy, rats were weighed to calculate the maternal weight gain (MWG) during pregnancy. After weighing on day 21 of pregnancy, rats were anesthetized with sodium thiopental (Thiopentax® - 50mg/kg) and exsanguinated for collection of maternal blood for

determination of heat shock proteins and antibodies. Laparotomy was then performed to remove the uterine horns for weighing of the litter. The ovaries were also removed and the corpora lutea counted and analyzed under a stereomicroscope. The numbers of implantations and live fetuses were counted to calculate the ratio of fetal viability (total number of live fetuses/total number of implantations).

Determination of hsp60 and hsp70 and respective antibodies

Serum hsp and antibodies were quantified in undiluted serum by commercial ELISA kits (hsp60 – IB09693 IBL-America; anti-hsp60 – IB09638 IBL-America; hsp70 – EKS-715 Assay Designs and Stressgen; anti-hsp70 – EKS-750 Assay Designs and Stressgen).

Placental and fetal weights

The placentas and term fetuses were removed and individually weighed. Each fetus was classified by the mean ± standard deviation (SD) according to the mean values of fetal weights of the non-diabetic group (ND): as small for pregnancy age (SPA) when weight was smaller than ND mean – 1.7x SD; appropriate for pregnancy age (APA) when weight was included in ND mean ± 1.7x SD; and large for pregnancy age (LPA) when weight was greater than ND mean + 1.7x SD (Soulimane-Mokhtari et al. 2005).

Analysis of visceral and skeletal malformations (MF)

Half of the fetuses were fixed in Bodian's solution and serial sections prepared as described by Wilson (1965) for visceral examination. The remaining fetuses were prepared for skeletal examination by the staining procedure of Staples and Schnell (1964). In addition to the skeletal analyses, counting of the ossification sites was performed according to the methodology of Aliverti (1979), which determines the degree of fetal development.

Statistical analysis

The normality of continuous variables was tested using the Kolmogorov and Smirnov's test. For variables normally distributed, data was analyzed using the One-way

Analysis of Variance (ANOVA) followed by the Tukey's Multiple Comparisons test. If the variables were not normally distributed, non-parametric statistical methods were used. To compare continuous non-parametric variables between multiple groups, the Kruskal–Wallis analysis of variance (ANOVA) by ranks test was performed. Fisher's exact test was used to compare proportions, and the Spearman correlation coefficient to assess the relationship between variables. A P value < 0.05 was considered significant.

RESULTS

The blood glucose levels during pregnancy are shown in Table 1. At day 0, day 7 and day 20, maternal blood glucose levels of MD rats were higher than levels of ND rats. During all stages of pregnancy SD rats presented with higher blood glucose levels compared to both ND and MD rats.

Data related to maternal reproductive performance are shown in Table 2. Rats from the MD group had reduced maternal weight gain (119g) and litter weight (84g) compared with ND rats (156g and 107g; $P<0.01$ and $P<0.05$, respectively). Rats from the SD group had reduced maternal weight gain (84g) and litter weight (63g) during pregnancy compared to both ND ($P<0.001$) and MD ($P<0.05$) groups. Moreover, the both MD and SD rats had a reduced number of implantations (11) and live fetuses (10) compared to the ND group (13; $P<0.05$ and $P<0.01$, respectively). In addition, the number of corpora lutea did not show differences among experimental groups. Both MD and SD groups had a reduced ratio of fetal viability (86% and 87%, respectively) compared to ND rats (97%; $P<0.0001$ and $P<0.001$, respectively).

We obtained 77 fetuses and placentas from ND rats, 151 from MD rats and 76 from SD rats (Table 2). Fetuses born from MD rats had reduced weight compared to fetuses from ND rats (5.2g vs. 5.4g, respectively; $P<0.05$). Similarly, fetuses from SD rats had reduced weight (4.3g) compared to both the ND and MD groups ($P<0.001$). The placentas of MD rats had reduced weight compared to ND rats (0.5g vs. 0.6g; $P<0.01$); and the placental weight of SD rats was higher (0.7g) compared to both the ND and MD groups ($P<0.01$).

Rats in the ND group had predominantly fetuses with appropriate weights for gestational age (APA – 92.7%). The remaining fetuses were evenly distributed between small for pregnancy age (SPA – 3.9%) and large for pregnancy age (LPA – 3.4%). In the MD group, 70.8% of fetuses were classified as APA, 19.0% as SPA and 10.2% as LPA. SD rats

had only 24.8% of fetuses with appropriate weight, 74.6% of fetuses were classified as small and 0.6% large for pregnancy age. There was a significant increase in SPA fetuses and reduction of APA fetuses in the MD and SD groups compared to the ND group ($P<0.0001$). The proportion of LGA fetuses in the MD group was significantly higher compared to the ND and SD groups ($P<0.0001$); comparing the last two groups, the SD group presented with a lower incidence ($P=0.011$) of large fetuses compared to ND fetuses (Fig. 1).

There was a significant increase in concentration of hsp60 in SD rats (2109 pg/mL) comparing to both the ND (1437 pg/mL) and MD (1486 pg/mL) groups ($P<0.0001$ and $P<0.001$, respectively). No significant difference in hsp60 protein levels was found between MD and ND rats (Fig. 2).

Likewise, the concentration of anti-hsp60 in the SD group (404 ng/mL) was significantly higher as opposed to the ND (172 ng/mL) and MD (198 ng/mL) groups ($P<0.001$). No significant difference in anti-hsp60 levels was found between MD and ND rats (Fig. 3).

There was a significant increase in concentration of hsp70 in SD rats (1301 pg/mL) comparing to both the ND (45.8 pg/mL) and MD (35.8 pg/mL) groups ($P<0.001$). No significant difference in hsp70 protein levels was found between MD and ND rats (Fig. 4).

Similarly, the concentration of anti-hsp70 in the SD group (1974 ng/mL) was significantly higher as opposed to the ND (718 ng/mL) and MD (961 ng/mL) groups ($P<0.05$). No significant difference in anti-hsp70 levels was found between MD and ND rats (Fig. 5).

The incidence of fetal visceral and skeletal malformations is shown in Table 3. Fetuses from MD rats presented only one type of malformation in higher incidence compared to fetuses from ND rats: enlarged trachea (14.3% vs. 0.0%, respectively; $P=0.0286$). As expected, several malformations were found in the SD group and some of these showed a

significant increase in comparison with the ND and/or MD groups: microphthalmia (P=0.0370), altered crystalline (P<0.0001), dilated renal pelvis (P=0.0121 and P=0.0068, respectively), enlarged ureter (P=0.0054 and P<0.0001, respectively), sinuous ureter (P=0.0497), and enlarged trachea (P=0.0055). There were no significant differences between the skeletal malformations found in fetuses from ND and MD rats (P>0.05). However, fetuses born from SD rats presented with a higher incidence of almost all skeletal malformations in comparison with both ND and MD rats: abnormally shaped sternebrae (P=0.0006 and P<0.0001, respectively), incomplete ossification of sternebrae (P=0.0024 and P=0.0039, respectively), supranumerary ribs, bipartite ossification of vertebral centrum, and abnormal ossification of vertebral centrum (P<0.0001).

The fetuses from SD rats showed a significantly reduced number of anterior (2.8) and posterior (0.3) phalanges, metatarsus (4.2), caudal vertebrae (4.0) and total ossification sites (21.2; P<0.001) compared with fetuses from ND and MD rats. In addition, fetuses from MD rats had reduced ossification in posterior phalanges (2.0), caudal vertebrae (4.6) and total ossification sites (24.9; P<0.01) compared with fetuses from ND rats (Table 4).

The relationship between heat shock proteins (hsp60 and hsp70) and their respective antibodies and maternal reproductive performance and fetal malformations is presented in Table 5 and Table 6, respectively. The hsp60 concentration was positively correlated with the level of anti-hsp60 (R=0.7493, P<0.0001). The same occurred between the levels of hsp70 and anti-hsp70 (R=0.4789, P=0.0154). We observed a positive correlation between both hsp and anti-hsp and blood glucose levels at all measured stages of pregnancy, except for the lack of an association between anti-hsp70 and blood glucose on day 20 of pregnancy (R=0.3245, P=0.0987). There was a positive correlation between hsp60 and hsp70 and the total number of malformations (R=0.5908, P=0.0024; R=0.4877, P=0.0134, respectively) and the number of malformations per fetus (R=0.6103, P=0.0015; R=0.4875, P=0.0134, respectively).

Antibodies to hsp were also positively correlated with malformations: anti-hsp60 and the number of malformations per fetus (R=0.3887, P=0.0451); and anti-hsp70 and the total number of malformations (R=0.3999, P=0.0387). Moreover, both hsp and anti-hsp showed very significant negative correlations with fetal weight.

DISCUSSION

To reproduce the hyperglycemia of human uncontrolled type-1 diabetes, experimental models have been developed to obtain the severe diabetic state (Eriksson et al. 2000, 2003; Rudge et al. 2007; Volpato et al. 2008; de Souza et al. 2009; Damasceno et al. 2011). In addition, models have been developed to reproduce the level of hyperglycemia typical of type-2 and gestational *Diabetes mellitus*. In laboratory animals, these latter types of diabetes are classified as mild diabetes (Portha et al. 1974; Tsuji et al. 1988; Oh et al. 1991; Caluwaerts et al. 2003; Soulimane-Mokhtari et al. 2005; Sinzato et al. 2009). In our study, as expected, SD rats had the highest, and MD rats had intermediate circulating blood glucose levels throughout pregnancy compared to non-diabetic rats.

In the present study, the SD rats tended to reduce the number of corpora lutea, evidence of a reduction in the number of oocytes liberated during the ovulation process. Likewise, these rats had a reduction in the number of implanted embryos and live fetuses as consequences of a hyperglycemic intrauterine environment. Diabetic rats, MD and SD, had a reduced maternal weight gain during pregnancy due to metabolic changes caused by hyperglycemia. Moreover, the same rats showed a reduced weight of their litter. The maternal weight gain and litter weight were reduced as blood glucose levels increased. In humans, inadequate maternal weight gain has been linked to an increased risk of delivery of a small-for-gestational-age infant (Cnattingius et al. 1998).

Regulation of fetal growth varies with the stage of gestation and is characterized by a major role for nutrient availability to the fetus and by the fact that the fetus and the placenta form a functional unit (Alsat et al. 1995). Placental structure and function can change as a result of maternal diabetes. In the present study, both MD and SD rats had altered placental and fetal weights. In MD group the placental weight was reduced and this can be associated to alterations in diabetes-derived placental cytoarchistructure (Sinzato et al. 2011). While the

placental weight in SD group was increased, which was interpreted as compensatory mechanism to intent to guarantee the maternal-fetal exchanges and nutrients supply to developing fetus. In spite of that, the hyperglycemia of maternal environment led to pancreas functional exhaustion of fetuses contributing for impaired growth and development (Calderon et al. 1992). The fetal weight classification showed increased rates of SPA fetuses, reduced rates of APA fetuses and decreased number of ossification sites of fetuses in both MD and SD groups, showing a delayed somatic development. The nature and extent of these changes depend on the type of diabetes and on the gestational period (Vambergue and Fajardy 2011). In humans, during the first trimester of pregnancy, embryonic growth might be controlled by nutrient supply and by locally active growth factors. Later, fetal growth depends essentially upon maternal-placental cooperation in delivering nutrients to the fetus. Fetal growth seems to be regulated by fetal insulin and insulin-like growth factors (IGF-1 and IGF-2), with growth hormone (GH) playing only a secondary role (Alsat et al. 1995).

Our findings of an increase in circulating hsp concentrations in SD rats with high levels of hyperglycemia corroborate previous studies. There are two reports of elevated serum hsp70 levels in type-1 diabetic patients (Oglesbee et al. 2005; Gruden et al. 2009). In another study, Hunter-Lavin et al. (2004) showed that serum hsp70 levels were higher in non-insulin treated type-2 diabetes subjects in comparison with insulin treated subjects, they concluded that hsp70 may therefore be a suitable marker of the severity of the clinical condition and may be useful in the monitoring of type-2 diabetes as well as other diseases associated with oxidative stress. Yabunaka et al. (1995) showed that levels of hsp70 in mononuclear lymphoid cells were significantly higher in diabetic patients compared with normal controls. Similarly, hsp60 and hsp70 were found to be induced in lymphocytes of patients suffering from type-2 diabetic nephropathy compared to controls (Calabrese et al. 2007). In contrast, other studies have reported reduced systemic hsp70 expression in

association with elevated glucose levels in both humans (Bruce et al. 2003; Chung et al. 2008) and non-human primates (Kavanagh et al. 2009). This apparent discrepancy remains to be resolved.

If hsp70 and hsp27 play a role in protecting embryonic cells from the cytotoxic effects of heat and developmental toxicants (Hale et al., 1996). Then, hsp70 levels are, possibly, increased as form for protection against hyperglycemia state in severe diabetic rats.

Increased concentrations of hsp70 in mononuclear cells of peripheral blood obtained from women in early pregnancy were associated with subsequent miscarriages, stillbirths and preterm births (Tan et al., 2007). Child et al. (2006) reported that serum anti-hsp70 antibody levels are significantly elevated at 16 weeks of gestation in women who later gave birth to babies with birth defects (cleft lip or palate or neural tube defects). As it was evidenced in our results, hsp70 level and its antibody were higher in severe diabetic rats, which also presented high rate of embryonic death and congenital malformation frequency. Further studies are required to clarify the association between circulating hsp70 and intrauterine growth restriction.

The higher incidence of fetal malformations, found in fetuses from SD rats in our study, is also consistent with previous investigations. As in human diabetic pregnancies, malformations in streptozotocin-induced experimental models of diabetes occur mainly in the neural system, heart, and skeleton (Eriksson 2009; Schaefer-Graf et al. 2000; Simán et al. 2000). Both increased oxidative stress and nitrosative stress are crucial features in diabetes-induced embryopathy and have been characterized in chemical-induced and genetic models of diabetes and even in mild diabetic experimental models (Eriksson et al. 2003; Jawerbaum and González 2006; Ornoy 2007). Impairment of the oxidative and nitrosative stress balance can deregulate multiple signaling pathways and cause massive cell damage, apoptotic events, and defective embryonic and fetal development (Sivan et al. 1997; Reece et al. 2005; Morgan

et al. 2008; Sugimura et al. 2009). In addition, the malformation rate is clearly correlated with increased glucose concentrations (Jawerbaum and White 2010). Experimental results support the notion of hyperglycemia as a teratogen, since high glucose levels (Dienelt and Zur Nieden 2010) or maternal diabetes in vivo as well as exposure to high glucose concentration cause embryonic maldevelopment. Several studies showed that fetuses from mild diabetic rats present with compromised intrauterine development (Saito et al. 2010; Iessi et al. 2010) and elevated oxidative stress, contributing to an increased incidence of skeletal and visceral malformations at birth (Damasceno et al. 2011).

Concentrations of hsp/anti-hsp in the present study were elevated in association with abnormal fetal weight and the occurrence of malformation. Belhia et al. (2010) showed that unexplained small fetuses for gestational age were positive for IgM and IgG antibody to human hsp60 and that the specific IgM antibody level was predictive of fetal mortality. The authors concluded that the detection of these antibodies indicated a placental perturbation and that a fetal autoimmune reaction to hsp60 was associated with this developmental delay. Child et al. (2006) have shown that anti-hsp70 levels were significantly elevated in patients who later gave birth to babies with cleft lip or palate or neurological abnormalities.

In summary, our results suggest that there is a relationship between levels of hsp60 and hsp70 and their respective antibodies and alterations in maternal reproductive performance and impaired fetal growth and development, evidenced by intrauterine growth restriction and fetal malformations in the context of different models of diabetes (levels of hyperglycemia). Further studies are required to determine the mechanisms that result in the increased levels of hsp and antibodies, and whether hsp have a direct role in the pathogenesis of congenital malformations and/or serve as biomarkers of altered reproductive outcome.

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TABLES AND FIGURES

Table 1. Blood glucose levels of dams throughout the pregnancy.

	ND	MD	SD
Glucose Mean (mg/dL)	89.33 ± 11.57	107.79 ± 23.95*	469.92 ± 68.03*$^{\#}$
Day 0 (mg/dL)	95.00 ± 7.97	112.54 ± 16.46*	484.13 ± 82.99*$^{\#}$
Day 7 (mg/dL)	100.67 ± 5.50	130.77 ± 11.89*	470.14 ± 39.82*$^{\#}$
Day 14 (mg/dL)	83.67 ± 9.52	94.23 ± 25.60	471.80 ± 35.69*$^{\#}$
Day 20 (mg/dL)	78.00 ± 6.81	93.62 ± 18.89*	445.00 ± 104.39*$^{\#}$

Blood glucose levels were measured on days 0, 7, 14 and 21 of pregnancy from non-diabetic (ND), mild diabetic (MD) and severe diabetic (SD) rats. Data are presented as mean ± standard deviation. *$P<0.05$ – compared to ND; $^{\#}P<0.05$ – compared to MD.

Table 2. Reproductive performance, fetal and placental weights of non-diabetic (ND), mild diabetic (MD) and severe diabetic (SD) rats at the end of pregnancy.

	ND	MD	SD
Number of corpora lutea	14.36 ± 2.44	14.00 ± 3.41	12.25 ± 2.49
Number of implantation	13.26 ± 2.82	11.44 ± 2.99*	10.88 ± 2.36*
Number of live fetus	12.82 ± 2.91	9.78 ± 3.40*	9.50 ± 2.78*
Fetal viability ratio (%)	96.67	85.50*	87.36*
Maternal weight gain (g)	155.80 ± 18.91	118.58 ± 23.83*	83.50 ± 11.67*$^{\#}$
Litter weight (g)	107.08 ± 19.51	83.75 ± 16.85*	62.72 ± 15.50*$^{\#}$
Fetal weight (g)	5.41 ± 0.43	5.17 ± 0.82*	4.34 ± 0.55*$^{\#}$
Placental weight (g)	0.57 ± 0.13	0.51 ± 0.10*	0.65 ± 0.10*$^{\#}$

Data are presented as mean ± standard deviation, except Fetal viability ratio, which is presented in percentage.

*P<0.05 – compared to ND; $^{\#}P$<0.05 – compared to MD.

Table 3. Incidence of visceral and skeletal malformations in fetuses from non-diabetic (ND), mild diabetic (MD) and severe diabetic (SD) rats.

MF (%)	ND	MD	SD
Number examined fetuses (visceral)	32	70	36
Total number of malformations	17	45	78
Enlarged lateral ventricle	0 (0.00)	1 (1.43)	0 (0.00)
Microphthalmia	0 (0.00)	0 (0.00)	3 (8.33)$^{\#}$
Altered crystalline	0 (0.00)	0 (0.00)	23 (63.89)*$^{\#}$
Dilated renal pelvis	0 (0.00)	2 (2.86)	7 (19.44)*$^{\#}$
Ectopic kidney	1 (3.13)	3 (4.29)	1 (2.78)
Enlarged ureter	6 (18.75)	9 (12.86)	19 (52.78)*$^{\#}$
Sinuous ureter	5 (15.63)	7 (10.00)	9 (25.00)$^{\#}$
Ectopic testis	0 (0.00)	2 (2.86)	0 (0.00)
Enlarged nasal cavity	0 (0.00)	4 (5.71)	3 (8.33)
Altered diaphragm	0 (0.00)	1 (1.43)	0 (0.00)
Enlarged trachea	0 (0.00)	10 (14.29)*	8 (22.22)*
Enlarged esophagus	0 (0.00)	3 (4.29)	2 (5.56)
Enlarged bronchus	0 (0.00)	3 (4.29)	3 (8.33)
Number examined fetuses (skeletal)	38	77	40
Total number of malformations	11	39	103
Abnormally shaped sternebrae	5 (13.16)	6 (7.79)	20 (50.00)*$^{\#}$
Incomplete ossif. sternebrae	0 (0.00)	3 (3.90)	9 (22.50)*$^{\#}$
Absent sternebrae	0 (0.00)	3 (3.90)	2 (5.00)
Supranumerary rib	5 (13.16)	13 (16.88)	35 (87.50)*$^{\#}$
Bipartite ossif. vert. centrum	0 (0.00)	4 (5.20)	14 (35.00)*$^{\#}$
Abnormal ossif. vert. centrum	1 (2.63)	10 (12.99)	23 (57.50)*$^{\#}$

Data are presented as count (percentage). *P<0.05 – compared to ND; $^{\#}P$<0.05 – compared to MD.

Legends: ossif, ossification; vert, vertebrae.

Table 4. Ossification sites of fetuses from non-diabetic (ND), mild diabetic (MD) and severe diabetic (SD) rats.

	ND	**MD**	**SD**
Anterior phalanges	3.96 ± 0.10	3.71 ± 0.42	2.77 ± 0.57*$^{\#}$
Metacarpus	4.00 ± 0.00	4.00 ± 0.00	3.93 ± 0.10
Posterior phalanges	3.38 ± 0.63	1.97 ± 0.96*	0.31 ± 0.59*$^{\#}$
Metatarsus	4.93 ± 0.12	4.66 ± 0.37	4.19 ± 0.20*$^{\#}$
Caudal vertebrae	5.48 ± 0.33	4.62 ± 0.50*	4.04 ± 0.24*$^{\#}$
Sternebrae	6.00 ± 0.00	5.94 ± 0.11	5.94 ± 0.11
Total	27.75 ± 0.97	24.90 ± 1.94*	21.18 ± 1.53*$^{\#}$

Data are presented as mean ± standard deviation. *P<0.05 – compared to ND; $^{\#}P$<0.05 – compared to MD.

Table 5. Relationship between heat shock proteins and maternal data and fetal malformations.

	Hsp60		**Hsp70**	
	R	*P*	*R*	*P*
Anti-hsp60	0.7493	<0.0001	-	-
Anti-hsp70	-	-	0.4789	0.0154
Glucose Day 0	0.7682	<0.0001	0.6991	0.0001
Glucose Day 7	0.7274	<0.0001	0.7226	<0.0001
Glucose Day 14	0.5919	0.0047	0.6425	0.0013
Glucose Day 20	0.7077	0.0003	0.6256	0.0018
Glucose Mean	0.8509	<0.0001	0.7363	<0.0001
Total MF	0.5908	0.0024	0.4877	0.0134
MF/Fetus	0.6103	0.0015	0.4875	0.0134
Fetal weight	-0.6859	0.0002	-0.6216	0.0009

Legends: MF, malformation.

Table 6. Relationship between antibodies to heat shock proteins and maternal data and fetal malformations.

	Anti-hsp60		**Anti-hsp70**	
	R	*P*	*R*	*P*
Hsp60	0.7493	<0.0001	-	-
Hsp70	-	-	0.4789	0.0154
Glucose Day 0	0.6694	0.0001	0.5337	0.0041
Glucose Day 7	0.6277	0.0006	0.4904	0.0094
Glucose Day 14	0.6305	0.0010	0.4089	0.0342
Glucose Day 20	0.5345	0.0071	0.3245	0.0987
Glucose Mean	0.6958	<0.0001	0.5116	0.0064
Total MF	0.3465	0.0766	0.3999	0.0387
MF/Fetus	0.3887	0.0451	0.3142	0.1104
Fetal weight	-0.6925	<0.0001	-0.4525	0.0178

Legends: MF, malformation.

Figure legends

Figure 1. Fetal weight classification. Fetal weight classification is defined as small for pregnancy age (SPA), appropriate for pregnancy age (APA) or large for pregnancy age (LPA).

Figure 2. Concentration of heat shock protein 60 kDa (hsp60) in serum from non-diabetic (ND), mild diabetic (MD) and severe diabetic (SD) rats. Data are presented as mean ± standard deviation. *P<0.05 – compared to ND; $^{\#}P$<0.05 – compared to MD.

Figure 3. Concentration of antibodies to heat shock protein 60 kDa (anti-hsp60) in serum from non-diabetic (ND), mild diabetic (MD) and severe diabetic (SD) rats. Data are presented as mean ± standard deviation. *P<0.05 – compared to ND; $^{\#}P$<0.05 – compared to MD.

Figure 4. Concentration of heat shock protein 70 kDa (hsp70) in serum from non-diabetic (ND), mild diabetic (MD) and severe diabetic (SD) rats. Data are presented as mean ± standard deviation. *P<0.05 – compared to ND; $^{\#}P$<0.05 – compared to MD.

Figure 5. Concentration of antibodies to heat shock protein 70 kDa (anti-hsp70) in serum from non-diabetic (ND), mild diabetic (MD) and severe diabetic (SD) rats. Data are presented as mean ± standard deviation. *P<0.05 – compared to ND; $^{\#}P$<0.05 – compared to MD.

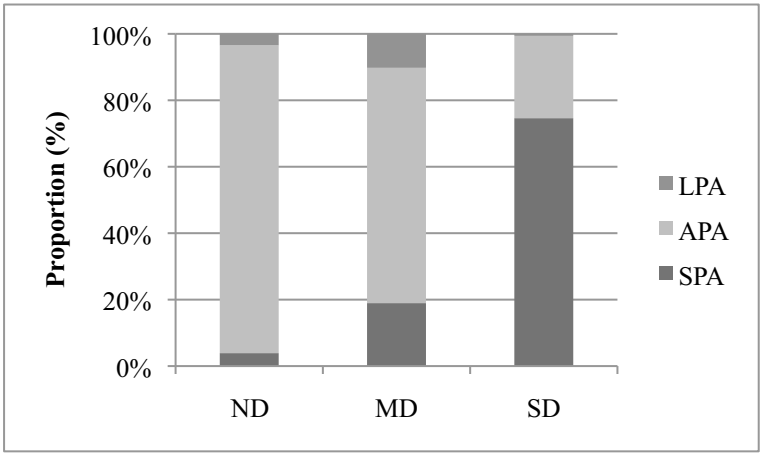

Figure 1. Fetal weight classification.

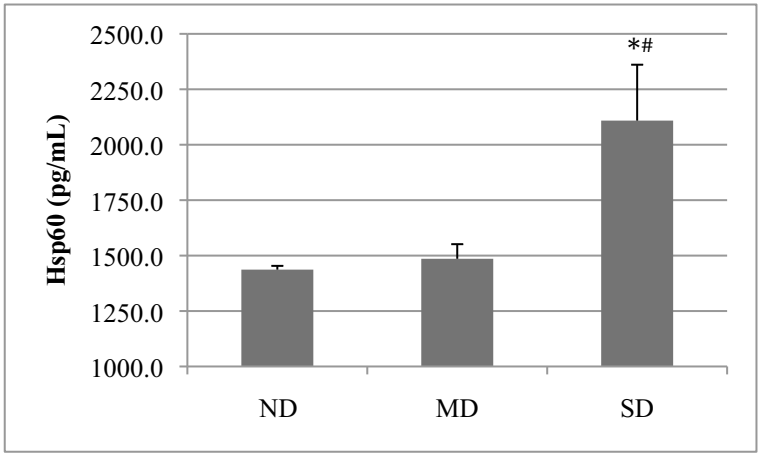

Figure 2. Concentration of heat shock protein 60 kDa (hsp60) in serum from non-diabetic (ND), mild diabetic (MD) and severe diabetic (SD) rats.

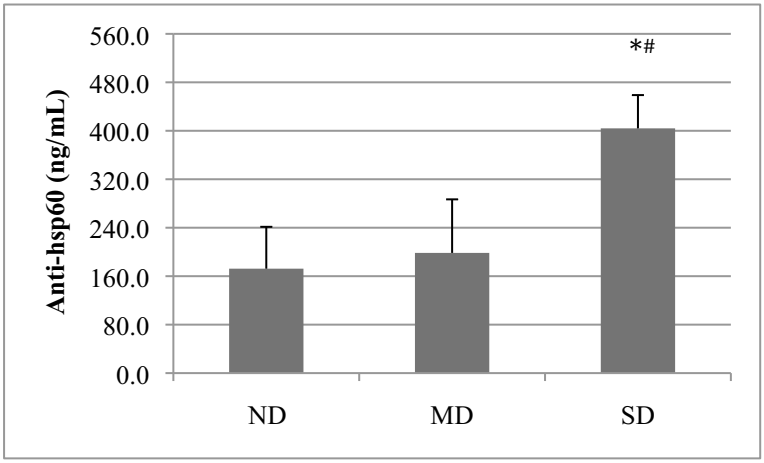

Figure 3. Concentration of antibodies to heat shock protein 60 kDa (anti-hsp60) in serum from non-diabetic (ND), mild diabetic (MD) and severe diabetic (SD) rats.

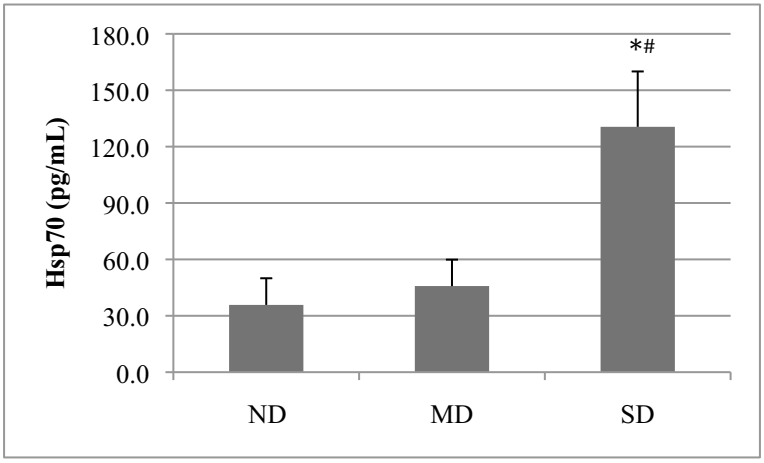

Figure 4. Concentration of heat shock protein 70 kDa (hsp70) in serum from non-diabetic (ND), mild diabetic (MD) and severe diabetic (SD) rats.

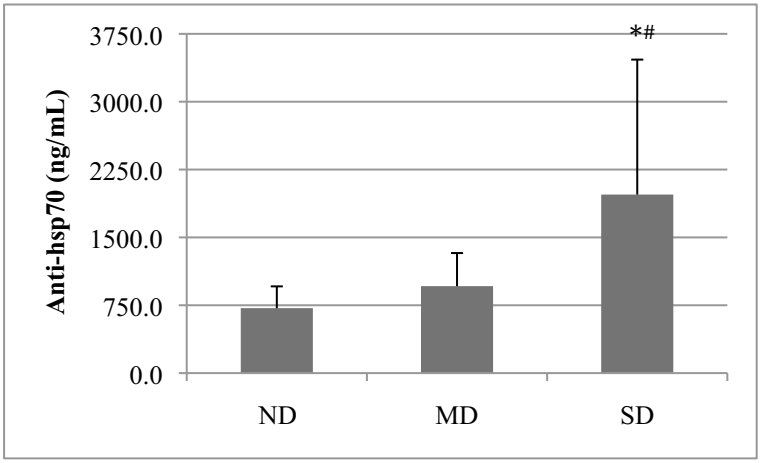

Figure 5. Concentration of antibodies to heat shock protein 70 kDa (anti-hsp70) in serum from non-diabetic (ND), mild diabetic (MD) and severe diabetic (SD) rats.

Anexos

FACULDADE DE MEDICINA
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Comissão de Ética em Experimentação Animal

Criada através da Portaria DFM nº 30 de 26/04/99

Certificado

Certificamos que o Protocolo n.º 787 sobre o projeto de pesquisa "Relação entre proteínas de choque térmico, reprodução materna e desenvolvimento fetal em diferentes modelos de diabete", de autoria de Felipe Hiroshi Saito, orientado pelo Prof. Dr. Steven Sol Witkin, com a participação de Aline Bueno, Aline de Oliveira Netto, Bruna Dallaqua, Debora Cristina Damasceno, Iracema de Mattos Paranhos Calderon, Isabela Lovizutto Iessi e Marilza Vieira Rudge, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), com a ressalva de que os "ratos", são provenientes de Biotério convencional, sem condições de atestar a Sanidade dos mesmos.

Projeto de Pesquisa aprovado em reunião da CEEA em 25/03/2010.

Profª Drª Regina H. Garcia Martins
Presidente da CEEA

Alberto Santos Capelluppi
Secretário da CEEA

Distrito Rubião Junior, s/nº - Botucatu – S.P. CEP: 18.618-970 Fone/Fax: (0xx14) 3811-6143 e-mail secretaria: capelluppi@fmb.unesp.br