Journal of Microbiology, Biotechnology and Food Sciences

Kádasi et al. 2012 : 1 (4) 488-497

REGULAR ARTICLE

THE EFFECT OF RAPAMYCIN ON SECRETORY ACTIVITY OF THE RABBIT OVARIAN FRAGMENTS

Attila Kádasi[1], Alexander V. Sirotkin[2], Nora Maruniaková[1], Sushmita Nath[3], Adriana Kolesárová[1]*

***Address**: [1]* Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, Department of Animal Physiology Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

[2]Institute for Genetics and Reproduction of Farm Animals, Animal Production Research Centre Nitra, Lužianky, Slovak Republic;

[3]Department of Life Science and Bioinformatics, Ethnobotany and Medicinal Plant Research Laboratory, Assam University, Silchar, India

Corresponding author*: attila.kadasi@gmail.com

ABSTRACT

The aim of our study was to examine the effect of rapamycin on secretory activity of the rabbit ovarian fragments. The secretion of steroid (progesterone, testosterone, estradiol) and peptide (prolactine) hormones by ovarian fragments after rapamycin addition at the doses 0, 1, 10, 100 $\mu g.ml^{-1}$ was determined. Fragments were incubated with rapamycin for 48 hours. Hormones were determinated by RIA. The experimental data showed that, addition of rapamycin did not affect progesterone and prolactine release (at all doses). Estradiol secretion was inhibited by rapamycin at the doses of 1, 10 and 100 $\mu g.ml^{-1}$. Testosterone was inhibited by the rapamycin at the doses of 1 and 10 $\mu g.ml^{-1}$ but not at 100 $\mu g.ml^{-1}$. In conclusion, our results suggest a direct effect of rapamycin on ovarian functionsand a possible involvement in the regulation of steroidogenesis.

Keywords: rapamycin, progesterone, testosterone, estradiol, prolactin, rabbit ovaries

INTRODUCTION

The mammalian target of rapamycin (mTOR) is a protein, serine/threonine protein kinase that regulates cell growth and proliferation, cell motility, protein synthesis and transcription **(Beevers *et al.*, 2006). M**TOR or Pathway of mTOR is an regulator of cell size that coordinates the activity of the cell growth machinery with the levels of energy and nutrients **(Sarbassov and Sabatini, 2005)**. It is cell signaling pathway, which is activated by steroid hormones and growth factors leading to cellular events including gene expression, cell proliferation and survival **(Makker *et al.*, 2011)**. When the genetic and environmental milieu is optimal for cellular growth, and diminishes under stressful conditions including insufficient nutrients, energy, or growth factors, as well as DNA damage actives mTOR signaling **(Kopelovich *et al.*, 2007)**. On the other hand rapamycin has potent immunosuppressive properties, it also has anticancer and antifungal activity inhibits the proliferation of fibroblasts, which leads to deterioration of wound healing **(Kahn *et al.*, 2005)**. It also inhibits abnormal cell proliferation and abnormal metabolism of cells **(Faivre *et al.*, 2006)**. Adding rapamycin to the diet increases life expectancy because it slows down the process of aging **(Powers *et al.*, 2006; Miller *et al.*, 2011)**. TOR inhibitor rapamycin was shown to increase life span in mice **(Harrison *et al.*, 2009)**. Rapamycin reduces number of estrus cycles, decreases size of preovulatory follicles and reduces in uterine size **(Shivaswamy *et al.*, 2011)**.

Progesterone is the ovarian steroid hormone that is needed for embryonic development and in mammary gland development **(Hagan *et al.*, 2009)**. It is produced by porcine ovarian granulosa cells **(Sirotkin *et al.*, 2008; Kolesarova *et al.*, 2010 a, b),** rabbit ovarian cells **(Sirotkin *et al.*, 2009),** corpus luteum of sheep **(Al-Dabbas *et al.*, 2008)** and goats **(Blaszczyk *et al.*, 2009)** and other animals. Progesterone governs ovarian functions of pigs **(Sirotkin *et al.*, 2008, Kolesarova *et al.*, 2010a,b)** and rabbits **(Sirotkin *et al.*, 2009)**. Testosterone is a steroid hormone that is produced in the testes of males, females in the ovaries and a small amount is produced by the adrenal gland **(Cox *et al.*, 2005; Reed *et al.*, 2006)**. It is important for healthy development of the individual and the establishment of secondary sexual characteristics **(Swaab *et al.*, 2009)**. Similarly, estradiol is one of the steroid hormones. It is produced by ovarian granulosa cells **(Rob *et al.*, 2008)**. To a lesser extent is also produced in the liver or adrenal cortex **(Nelson and Bulun, 2001)**. This hormone is responsible for the development of secondary sex characteristics **(Hess *et al.*, 1997)**. Luteotrophic hormone or prolactin, peptide hormone **(Bartholomew *et al.*, 2007),** is produced by the pituitary gland **(Sabharwal *et al.*, 1992)**. This hormone stimulates the enlargement of

the mammary glands during pregnancy, which is primarily associated with the process of lactation in mammals **(Bartholomew *et al.*, 2007)**.

The aim of this *in vitro* study was to investigate the influence of rapamycin on the secretion of steroid (progesterone, estradiol, testosterone) and peptide (prolactin) hormones from rabbit ovarian fragments.

MATERIAL AND METHODS

Ovaries were obtained from noncyclic rabbits of hybrid line New Zealand White aged 3.5 to 4 months in Animal Production Research Centre in Nitra. Ovaries were transported to the laboratory in containers at 4ºC and washed in sterile physiological solution and were sectioned in 8-16 fragments (approx. 2-3 mm size). Subsequently, these fragments (1 fragment per a well) were incubated in culture plates (Nunc™, Roskilde, Denmark, 1 ml.well^{-1}) with 1 ml of sterile culture medium DMEM/F12 1:1 (BioWhittakerTM, Verviers, Belgium) supplemented with 10% fetal calf serum (BioWhittakerTM) and 1% antibiotic - antimycotics (Sigma, St. Louis, MO, USA) with the addition of rapamycin (Fermentek Ltd., Jerusalem, Israel) at the doses of 0, 1, 10, 100 μg.ml^{-1} at 37 ° C, 5% CO_2 for 48 hours. After cultivation of fragments the culture medium was taken from wells plates by syringe and stored at -70°C for radioimmunoassay (RIA). The concentrations of progesterone, estradiol, testosterone and prolactin were determined by RIA in 25-100 ml of culture medium. These substances have been linked using RIA kits (Immunotech SAS, Marseille Cedex, France) according to manufacturer's instructions (Makarevich and Sirotkin, 1999). Assays of hormone levels in the culture media were performed in duplicate. The rates of substance secretion were calculated per mg tissue per day. Differences between groups were evaluated using t-test using statistical software Sigma Plot 11.0 (Janda, Corte Madera, USA). Values represent the mean ± SEM. Differences were compared for statistical significance at the P - level less than 0.05 ($P<0.05$).

RESULTS

Secretion of progesterone by ovarian fragments was not affected after the addition of rapamycin at the doses 1, 10 and 100 μg.ml^{-1} (Fig. 1 A). On the other hand significant ($P<0.05$) decrease of estradiol secretion was found after rapamycin addition at the doses 1, 10 and 100 μg.ml^{-1} (Fig. 1 B). Similarly, testosterone secretion was significantly ($P<0.05$)

inhibited by rapamycin at the doses of 1 and 10 µg.ml^{-1} but not at 100 µg.ml^{-1} (Fig. 1 C). Prolactin secretion was not affected by rapamycin at all doses (Fig. 1 D).

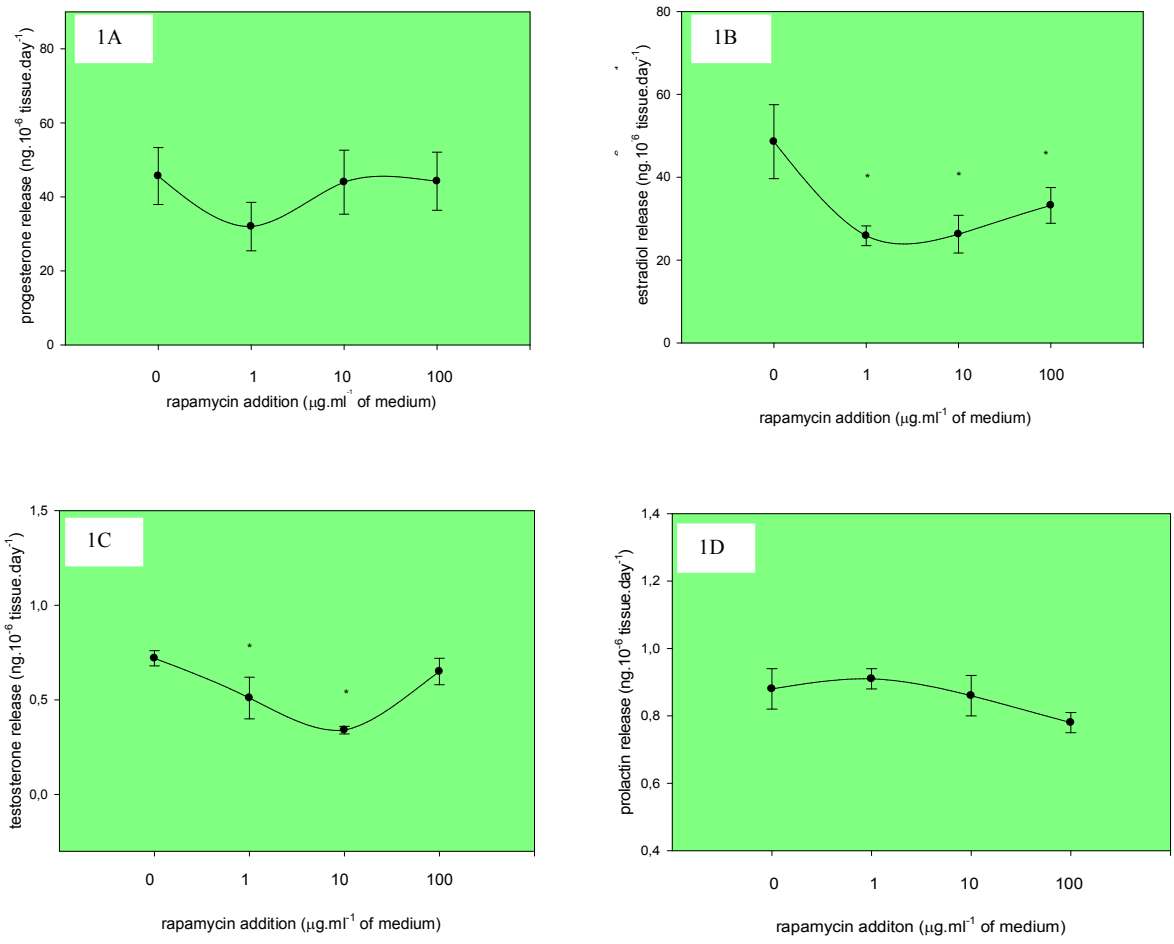

Figure 1 A-D Effect of rapamycin on steroid and peptide secretion by rabbit ovarian fragments. (A) progesterone (B), estradiol, (C) testosterone and (D) prolactin. *****Significant ($P<0.05$) differences compared to control group. Differences between groups were assessed t-test. Values represent the mean ± SEM. RIA.

DISCUSSION

The possible effect of rapamycin addition on ovarian functions of rabbits is suggested in this study. These data confirm the previous reports concerning the influence of rapamycin on bovinne **(Hou *et al.*, 2010)**, rat **(Wera *et al.*, 1995)**, mouse **(Yu *et al.*, 2011)** and human **(Kaczmarek *et al.*, 2004; Fritsche *et al.*, 2004)** cell processes.

A dose-dependent effect of rapamycin on progesterone secretion was not found. Similarly, in previous study rapamycine additon (20 nM) did not reduce luteinizing hormone (LH)-induced progesterone production in bovine luteal cells **(Hou *et al.*, 2010)**. The effect of rapamycin on the secretion of progesterone was not found.

In the study secretion of estradiol by ovarian rabbit fragments was decreased by rapamycin at all doses. The other authors described that at a reduced dose of rapamycin found a significant increase ($P<0.05$) of estradiol production 10 $ng.ml^{-1}$ in mouse follicles **(Yu *et al.*, 2011)**. **Sanchez *et al.* (2011)** established by immunoblotting that estradiol antagonized the effect of everolimus, another mTOR inhibitor system. **Ray *et al.* (2011)** found that repeated addition of estradiol did not affect the inhibition of proliferation by rapamycin. Our findings suggest that rapamycin is a possible inhibitor of secretion of estradiol and the process of steroidogenesis in rabbit ovaries.

A dose-dependent effect of rapamycin on testosterone secretion was found. Testosterone was inhibited by rapamycin addition at the doses of 1 and 10 $\mu g.ml^{-1}$ but not at 100 $\mu g.ml^{-}1$. **Kaczmarek *et al.* (2004)** established mean testosterone release was 3.86 ± 1.41 $ng.ml^{-1}$ in the sirolimus group gonadal functions of men and 4.55 ± 1.94 $ng.ml^{-1}$ in the controls ($P=0.025$). **Fritsche *et al.* (2004)** has found that testosterone values were lower (11.2 ± 6.3 $nmol.l^{-1}$ vs. 15.5 ± 7.7 $nmol.l^{-1}$, ($P<0.05$), in sirolimus-treated patients compared to non-sirolimus-treated controls**.** The findings of **Skrzypek and Krause (2007)** were in accordance with the previous studies. The authors have confirmed the reduction of testosterone level by rapamycin addition. **Wu *et al.* (2010)** established the inhibition of mTOR activity by rapamycin on human cancer cells, but it was not dependent on testosterone concentration. The previous reports and our findings confirm the inhibitory effect of rapamycin on the secretion of testosterone and the process of steroidogenesis in rabbit ovaries.

Secretion of peptide hormone prolactin by ovarian fragments was not affected by rapamycin addition at all doses used in our study. Similarly, **Wera *et al.* (1995)** established rapamycin addition did not have the effect on the prolactin release measured during a 2h incubation period, indicating that they do not influence the secretion of prolactin from intracellular stores into the culture medium. During longer incubation times (48 h), however, prolactin release was diminished to 64% ±14 (1 μM rapamycin), suggesting an effect on prolactin production of rats. **Fritsche *et al.* (2004)** found that values of prolactin levels were not different in sirolimus-treated patients compared to non-sirolimus-treated controls**.** **Belkowski *et al.* (1999)** described rapamycin markedly inhibited proliferation and prolactin

translocation to the nucleus of cloned murine. The effect of rapamycin on the secretion of prolactin was not confirmed.

CONCLUSION

The present study describes the possible effect of rapamycin on rabbit ovarian functions. The results of this study suggest a possible dose-dependent effect of rapamycin on the secretory activity of some steroid hormones (estradiol, testosterone but not progesterone) secreted from the ovarian fragments of rabbits. The effect of the mTOR inhibitor has been confirmed at the doses of 1, 10 and 100 $\mu g.ml^{-1}$ on estradiol secretion and at 1 and 10 $\mu g.ml^{-1}$ on testosterone secretion. In conclusion, our results suggest a direct effect of rapamycin on ovarian functions and a possible involvement in the regulation of steroidogenesis in rabbit ovaries.

Acknowledgments: We would like to thank Mrs. Katarína Tóthová and Ing. Žofia Kuklová, the employees of RIA laboratory of Animal Production Research Centre in Nitra. This work was financially supported by VEGA scientific grant no. 1/0790/11 and 1/0084/12.

REFERENCES

AL-DABBAS, F. M.- HAMR, A. H. - AWAWDEH, F. T. 2008. The effect of arginine supplementation on some blood parameters, ovulation rate and concentrations of estrogen and progesterone in female Awassi sheep. In *Pakistan Journal of Biological Science*, vol. 11, p. 2389-2394.

BARTHOLOMEW, E. F. - MARTINI, F. - OBER, W. B. 2007. Essentials of anatomy & physiology. San Francisco: Pearson/Benjamin Cummings, p. 340. ISBN 0-8053-7303-9.

BEEVERS, C. - LI, F. - LIU, L. - HUANG, S. 2006. Curcumin inhibits the mammalian target of rapamycin-mediated signaling pathways in cancer cells. In *International Journal of Cancer,* vol. 119, 2006, no. 4, p. 757–764..

BELKOWSKI, S. M. - LEVINE, J. E. - PRYSTOWSKY, M. B. 1999. Requirement of PI3-kinase activity for the nuclear transport of prolactin in cloned murine T lymphocytes. In *Journal of Neuroimmunology,* vol. 94, 1999, no. 1-2, p. 40-47.

BLASZCZYK, B. - STANKIEWICZ, T. - UDALA, J. - GACZARZEWICZ, D. 2009. Plasma progesterone analysis by time-resolved fluorescent antibody test to monitor estrous cycles in goats. In *Journal of Veterinary Diagnostic Investigations*, vol. 21, 2009, p. 80-87.

COX, R. M. - JOHN-ALDER, H. B. 2005. Testosterone has opposite effects on male growth in lizards (Sceloporus spp.) with opposite patterns of sexual size dimorphism. In *Journal of Experimental Biology,* vol. 208, 2005, no. 24, p. 4679-4687.

FAIVRE, S. - KROEMER, G. - RAYMOND, E. 2006. Current development of mTOR inhibitors as anticancer agents. In *Nature Reviews Drug Discovery,* vol. 5, 2006, p. 671-688

HAGAN, C. R. - FAIVRE, E. J. - LANGE, C. A. 2009. Scaffolding actions of membrane-associated progestrerone receptors. In *Steroids,* vol. 74, 2009, p. 568-572.

HARRISON, D. E. - STRONG, R. - SHARP, Z. D. - NELSON, J. F. - ASTLE, C. M. - FLURKEY, K. - NADON, N. L. - WILKINSON, J. E. - FRENKEL, K. - CARTER, C. S. - PAHOR, M. - JAVORS, M. A. - FERNANDEZ, E. - MILLER, R. A. 2009. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. In *Nature,* vol. 460, 2009, p. 392–395.

HESS, R. A. - BUNICK, D. - LEE, K. H. - BAHR, J. - TAYLOR, J. A. - KORACH, K. S. - LUBAHN, D. B. 1997. A role for oestrogens in the male reproductive system. In *Nature,* vol. 390, 1997, p. 447-8.

HOU, X. - ARVISAIS, E. W. - DAVIS, J. S. 2010. Luteinizing Hormone Stimulates Mammalian Target of Rapamycin Signaling in Bovine Luteal Cells via Pathways Independent of AKT and Mitogen-Activated Protein Kinase: Modulation of Glycogen Synthase Kinase 3 and AMP-Activated Protein Kinase. In *Endocrinology*, vol. 151, 2010, no. 6, p. 2846-2857.

HUANG, S. - BJORNSTI, M. - HOUGHTON, P. 2003. Rapamycins: mechanism of action and cellular resistance. In *Cancer Biology & Therapy,* vol. 2, 2003, no. 3, p. 222–232.

KACZMAREK, I., GROETZNERA, J., ADAMIDIS, I., LANDWEHR, P., MUELLER, M., VOGESERB, M., GERSTORFER, M., UBERFUHR, P., MEISER, B., REICHART, B. 2004. Sirolimus Impairs Gonadal Function in Heart Transplant Recipients. In *American Journal of Transplantation,* vol. 4, 2004, p. 1084–1088.

KAHN, D., SPEARMAN, C.W., MALL, A., SHEPHERD, E., ENGELBRECHT, G., LOTZ, Z., TYLER, M. 2005**.** Effect of Rapamycin on the Healing of the Bile Duct. In *Transplantation Proceedings,* vol. 37, 2005, issue 2, p. 832-833.

KOLESAROVA, A., ROYCHOUDHURY, S., SLIVKOVA, J., SIROTKIN, A., CAPCAROVA, M., MASSANYI, P. 2010: In vitro study on the effect of lead and mercury on

porcine ovarian granulosa cells. In *Journal of Environmental Science and Health, Part A Toxic/ Hazard Substances Environmental Engeneering,* vol. 45, 2010a, p. 320-331.

KOLESAROVA, A., CAPCAROVA, M., SIROTKIN, A., MEDVEDOVA, M., KOVACIK, J. 2010. Cobalt-induced changes in the IGF-I and progesteron release, expression of proliferation- and apoptosis-related peptides in porcine ovarian granulosa cells in vitro. In *Journal of Environmental Science and Health, Part A Toxic/ Hazard Substances Environmental Engeneering,* vol. 45, 2010b, p. 810-817.

KOPELOVICH, L., FAY, J.R., SIGMAN, C. C., CROWELL, J. A. 2007. The Mammalian Target of Rapamycin Pathway as a Potential Target for Cancer Chemoprevention. In *Cancer Epidemiology, Biomarkers and Prevention,* vol. 16, 2007, issue 7, p. 1330-1340.

MAKAREVICH, A., SIROTKIN, A., 1999. Development of sensitive radioimmunoassay for IGF-I determination in samples from blood plasma and cell-conditioned medium. In *Veterinarna medicina*., vol. 44, 1999, p. 71-78.

MAKKER, A., GOEL, M. M., DAS, V., AGARWAL, A. 2011. PI3K-Akt-mTOR and MAPK signaling pathways in polycystic ovarian syndrome, uterine leiomyomas and endometriosis: an update. In *Gynecological Endocrinology,* vol. Sept.,2011, p. 1-7.

MILLER, R. A., HARRISON, D. E., ASTLE, C. M., BAUR, J. A., BOYD, A. R., DE CABO, R., FERNANDEZ, E., FLURKEY, K., JAVORS, M. A., NELSON, J. F., ORIHUELA, C. J., PLETCHER, S., SHARP, Z. D., SINCLAIR, D., STARNES, J. W., WILKINSON, J. E., NADON, N. L., STRONG, R. 2011. Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice. In *Journal of Gerontology, Series A: Biological Sciences and Medical Sciences,* vol. 66, 2011, issue 2, p. 191-201.

NELSON, L. R., BULUN, S. E. 2001. Estrogen production and action. In *Journal of the American Academy of Dermatology,* vol. 45, 2001, p. 116–24.

POWERS, R. W., KAEBERLEIN, M., CALDWELL, S. D., KENNEDY, B. K., FIELDS, S. 2006. Extension of chronological life span in yeast by decreased TOR pathway signaling". In *Genes and Developement,* vol. 20, 2006, issue 2, p. 174–84.

RAY, S., FRY, M. J., DARBRE, P. D. 2011.Enhanced sensitivity to rapamycin following long-term oestrogen deprivation in MCF-7, T-47-D and ZR-75-1 human breast cancer cells. In *The Journal of Endocrinology,* vol. 208, 2011, issue 1, p. 21-29.

REED, W. L., CLARK, M. E., PARKER, P. G., RAOUF, S. A., ARGUEDAS, N., MONK, D. S., SNAJDR, E., NOLAN, V., KETTERSON, E. D. 2006. Physiological effects on demography: a long-term experimental study of testosterone's effects on fitness. In *The American Naturalist,* vol. 167, 2006, no. 5, p. 667–83.

ROB, L., MARTAN, A., CITTERBART, K. et al. Gynekologie. 2. vyd. Praha : Galen, 2008, 319 p. ISBN 978-80-7262-501-7.

SABHARWAL, P., GLASER, R., LAFUSE, W., VARMA, S., LIU, Q., ARKINS, S., KOOIJMAN, R., KUTZ, L. et al. 1992. Prolactin synthesized and secreted by human peripheral blood mononuclear cells: an autocrine growth factor for lymphoproliferation. In *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, 1992, no. 16, p. 7713–7716.

SANCHEZ, C. G., MA, C. X., CROWDER, R. J., GUINTOLI, T., PHOMMALY, C., GAO, F., LIN, L., ELLIS, M. J. 2011. Preclinical modeling of combined phosphatidylinositol-3-kinase inhibition with endocrine therapy for estrogen receptor-positive breast cancer.In *Breast Cancer Research,* vol. 13, 2011, issue 2, R21.

SARBASSOV, D. and SABATINI, M. 2005. Redox Regulation of the Nutrient-sensitive Raptor-mTOR Pathway and Complex. In *The journal of biological chemismy,* vol. 280, 2005, no.47, p. 39505–39509.

SHIVASWAMY, V., OCHSNER, L., MARONI, D., WANG, C., PASSER, J., CLURE, C. E., HAMEL, F. G., DAVIS, J. S., LARSEN, J. 2011. Tacrolimus and sirolimus induce reproductive abnormalities in female rats. In *Transplantation*. vol. 91, 2011, issue 12, p. 1333-1339.

SCHWAB, M. S., KIM, S. H., TERADA, N., EDFJÄLL, C., KOZMA, S. C., THOMAS, G., MALLER, J. L. 1999. $p70^{S6K}$ Controls Selective mRNA Translation during Oocyte Maturation and Early Embryogenesis in *Xenopus laevis.* In *Molecular and Cellular Biology*, vol. 19, 1999,no. 4, p. 2485-2494.

SIROTKIN, A. V., BENCO, A., TANDLMAJEROVA, A., VASICEK, D., KOTWICA, J., DARLAK, K. 2008. Transcription factor p53 can regulate proliferation, apoptosis and secretory activity of luteinizing porcine ovarian granulosa cell cultured with and without ghrelin and FSH. In *Reproduction,* vol.136, 2008, p. 611-618.

SIROTKIN, A. V., CHRENEK, P., DARLAK, K., VALENZUELA, F., KUKLOVA, Z. Some endocrine traits of transgenic rabbits. II. Changes in hormone secretion and response of isolated ovarian tissue to FSH and ghrelin. In *Physiological Research,* vol. 57, 2008, p. 745-751.

SIROTKIN, A. V, RAFAY, J, KOTWICA, J. Leptin controls rabbit ovarian function in vivo and in vitro: possible interrelationships with ghrelin. In *Theriogenology,* vol. 72, 2009, no. 6, p. 765-72.

SKRZYPEK, J., KRAUSE, W. 2007. Azoospermia in a renal transplant recipient during sirolimus (rapamycin) treatment. In *Andrologia,* vol. 39, 2007, p. 198–199.

SWAAB, D. F., GARCIA-FALGUERAS, A. 2009. Sexual differentiation of the human brain in relation to gender identity and sexual orientation. In *Functional Neurology,* vol. 24, 2009, no.1, p. 17–28.

WERA, S., ZHENG, L., HOOGHE-PETERS, E. L., BELAYEW, A., MARTIAL, J. A., VELKENIERS, B. 1995. Cyclosporin A, rapamycin and FK506 decrease prolactin release from rat pituitary cells in primary culture. In *Endocrine Reserarch,* vol. 21, 1995, issue 3, p. 623-33.

WU, Y., CHHIPA, R. R., CHENG, J., ZHANG, H., MOHLER, J. L., CLEMENT, P. 2010. Androgen Receptor-mTOR Crosstalk is Regulated by Testosterone Availability: Implication for Prostate Cancer Cell Survival. In *Anticancer Research,* vol. 30, 2010, p. 3895-3902.

YU, J., YABA, A., KASIMAN, C., THOMSON, T., JOHNSON, J. 2011. mTOR Controls Ovarian Follicle Growth by Regulating Granulosa Cell Proliferation. In *PLoS ONE,* vol. 6, 2011, issue 7, p.1-10.