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Pre-Implantation Immunosuppression in the Pregnant Rat: A Possible Role of Seminal Fluid

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كبت المناعة قبل الغرس الجنيني في أنثى الجرذ الحامل : دور محتمل للسائل المنوي

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خلاصة : تتعرض الأنثى خلال عملية التناسل الطبيعي إلى تحدٍّ مناعي عكسي . وتتطرق هذه الدراسة البحثية إلى دراسة النظرية التي تقترح بأن لدى السائل المنوي إمكانات مناعية مخفضة خلال المراحل الأولية للحمل ، والتي يمكن بواسطتها الحماية المبكرة للجنين من تأثيرات مناعية معاكسة تضر بالحمل . وتستخدم هذه الدراسة إحدى عشرة مجموعة من الجرذان المخبرية من فصيلة (AO(RT1^u مقسمة إلى ثلاث مجموعات . المجموعة الأولى كانت خلال يوم الفترة الودقية (الطمث) للدورة المبيضية وذلك للحصول على المعلومات الاساسية والمبدئية لهذه الدراسة . والمجموعة الثانية تمت دراستها يومياً خلال الأيام الخمس الأولى لفترة الحمل الكاذب ، ولقد اعتبرت هذه المجموعة كمقياس ضبط أساسي لهذا البحث . أما المجموعة الثالثة من إناث الجرذان فلقد تمت دراستها يومياً خلال الأيام الخمس الأولى من الحمل الطبيعي المتماثل خلقياً ، وحدث ذلك بتزاوجها مع ذكور متماثلة جينياً معها . ولقد احتوت كل مجموعة على ستة جرذان على الأقل . وتم استحصال أوزانها وأوزان أنسجتها التي تضمنت الرحم والغدة الزعترية (الثيموسية) والعقد الليمفاوية الرجوية للرحم والعقد المأبضية . وجمعت هذه الأنسجة يومياً خلال فترة الودق والأيام الخمسة الأولى للحمل والحمل الكاذب . وتدعم نتائج هذه الدراسة نظرية الهبوط المناعي الناتج عن وجود السائل المنوي خلال الفترة المبكرة للحمل ، والذي يؤدي إلى حدوث ردود فعل مماثلة في انقسامات الخلايا الليمفاوية المتواجدة في العقد الليمفاوية الرجوية للرحم .

ABSTRACT: Successful reproduction involves exposure of the female to significant antigenic challenge. These experiments were done to test the possibility/hypothesis that seminal fluid has an immunosuppressive role in the early stages of pregnancy, which could protect the conceptus from immunological attack. A highly inbred strain of rat AO(RT1^u) was used in this study. Eleven groups of animals were examined, one during the estrous phase of the ovarian cycle to provide base-line data, five during pseudopregnancy, designated as controls, and five groups during syngeneic pregnancy. Each group contained a minimum of six animals. For each animal, body weight and organ/tissue weights of the uterus, thymus, uterine and popliteal lymph nodes, were recorded together with total cell counts of the lymphoid tissues and their proliferating cell counts. The material was collected during each of the first five days of pregnancy and pseudopregnancy. The present study supports an immunological suppression by seminal fluid during early pregnancy as shown by a significant sustained depression of the proliferative lymphocyte response in the uterine regional lymph nodes.

The antigenicity of spermatozoa is well established (Mori et al., 1990; Ohashi et al., 1990). Seminal fluid comprises approximately 80-90% of the volume of the normal ejaculate. It is a mixture of secretions from various parts of the male reproductive tract and its associated structures. Certain components of seminal fluid are thought to assist the process of fertilization; constituent enzymes facilitate spermatozoal transport through the female reproductive tract and are considered essential for oocyte penetration, while fructose provides an effective energy source for spermatozoal motility (James and Hargreaves, 1984). In addition, seminal fluid contains a number of immunosuppressive factors which may protect spermatozoa from immunological damage and prevent sensitization of the female to spermatozoal antigens following coitus (Stites and Erickson, 1975, Quayle and James, 1990). During transit through the epididymis and the vas deferens spermatozoa become coated with seminal fluid components which render them much less immunogenic (Prakash and Lang, 1980; Anderson and Tarter, 1982). The reduction of spermatozoal immunogenicity by seminal fluid may also suppress other immunological reactions in the recipient of the ejaculate and this may have undesirable effects, especially locally.

At coitus, some spermatozoa penetrate the uterine mucous membrane (Genin, 1953) and large numbers invade the endometrial glands (Hafez, 1976). Radiolabelled epididymal spermatozoa introduced into the uterine lumen were found 17 hours later in the endometrium, pelvic lymph nodes and spleen (Reid, 1965*a,b*). Subcutaneous challenge and artificial

insemination with allogeneic epididymal spermatozoa generates specific anti-male strain cytolytic T-lymphocytes in the regional lymph nodes of the female recipient (Thomas et al., 1981; Thomas and McLean, 1984) which continues until the 5th day, while after the first allogeneic mating it is limited to the 2nd and 3rd post coital day, being no longer detectable at the time of implantation on the 5th post-coital day. This 'switch-off' of the cytolytic T-cell response to allogeneic mating during the pre-implantation period suggests that some factor in the seminal fluid suppresses the female host's immune response to spermatozoal alloantigens. Whilst a limited cytolytic T-cell response to coitus resulting from the effects of seminal fluid is clearly beneficial in reproduction, it may have adverse effects if the host is exposed to a virally infected ejaculate. Since the host's defence against viral infection is predominantly cell-mediated this may be clinically important since there is now a well-established link between human papilloma virus infection and cervical neoplasia (Reid, 1984; Yee et al., 1985; Schwarz et al., 1985).

Materials and methods

A highly inbred strain of rat AO(RT1^u/AgB2), was used in this investigation. The sexually mature females were bred from a specially maintained stock on a regular diet and with a 12 hours light cycle. At the time of sacrifice, the rats had a body weight of 150 g or more and were between 13 and 15 weeks of age in order to minimize age-related changes in the lymphoid tissues. In the sexually mature rat, the various stages of the estrous cycle can be determined by microscopy of the unstained smear taken daily with a wire loop from the lower vagina (Stockard and Papanicolau, 1917). Although the rat cycle is subject to individual variations, the estrous phase recurs every 4-5 days in the absence of mating (Long and Evans, 1922).

Eleven groups of animals were used in this investigation; 5 groups designated as controls were examined during pseudopregnancy, and 5 groups during syngeneic pregnancy. The remaining group provided data on estrous phase virgin animals; day 0 of the observation period. This group, designated group 0 provided base-line data for both pseudopregnant and pregnant animals, since the initiation of pseudopregnancy or pregnancy took place on that day.

Pseudopregnancy was induced in virgin rats by mechanical cervical stimulation during estrus. An electrical vibrator was used and stimulation was maintained for a minimum of 30 seconds in the restrained animal (DeFeo, 1966). The day following cervical stimulation was designated the first day of pseudopregnancy. The pseudopregnant groups, designated 1-5, were sacrificed at 24 hour intervals after the vaginal smear had confirmed a diestrous type smear. In the m (pregnant) groups, 1 to 3 mature female rats in e were caged late in the afternoon with a male rat of pr fertility. Vaginal smears were prepared from the ra 10.00 hours each day. The day spermatozoa were not the smear was designated day 1 of pregnancy. remaining groups, designated 2 to 5, were sacrificed hour intervals following day 1 of pregnancy. All an groups were sacrificed at approximately 11.00 hours, verification of the diestrous vaginal smear.

Each group contained at least six animals and animal yielded figures for body weight and organ/ti weights for the uterus, thymus, uterine regional ly nodes (URLN) and popliteal lymph nodes. Each lymp tissue, provided data for total lymphocyte content density and percentage of radio-labelled cells.

On the day of sacrifice, each animal was anesthet with ether and given an i.p. injection of 12 m veterinary Nembutal ('Sagatal': May & Baker Dagenham). The weight of the animal was recorded after exsanguination the uterus, thymus, uterine regi lymph nodes and popliteal lymph nodes were remo and cleaned. The uterus was opened along its length weighed, while the lymphoid tissues were weighed placed in '199' tissue culture medium (Wellc Reagents Ltd., Beckenham) adjusted to pH 7.2 . A suspension from each of the lymphoid tissues prepared by repeated squashing. Each cell suspension subsequently filtered through a stainless steel mesh holes/cm^2) and made up to a final volume of 10 ml fo thymus and 5 ml for each lymph node group; 1 ml taken from each of these final volumes and used for cell counts, using a Coulter ZB counter.

After removal of aliquots for the cell counts 10 [6-^{3}H] thymidine (Amersham International Aylesbury) at an activity of 100 µCi/ml were adde each 1 ml of cell suspension. After mixing, suspensions were incubated in a water bath at 37 °C 1 hour with occasional shaking. The suspensions then centrifuged at 2000 rpm (700 g) for 10 minutes the resultant pellets resuspended in phosphate buf saline at a concentration of 5 X 10^6 cells/ml. Pre work has shown that at this dilution the cell suspe requires no further washing. This cell concentrati also optimal for preparing smears in the Shandon- cytocentrifuge. After radiolabelling, six smears prepared from each lymphocyte suspension. The s were prepared on slides previously coated gelatin/chrome alum (Rogers, 1969) to prevent peel the nuclear emulsion used in the autoradiograph smears were dried in air and fixed in methanol, dip diluted Ilford K2 nuclear emulsion (Ilford Knutsford) and stored at 4 °C in the dark for three After this period, when a trial slide showed d labelling, the remaining slides from that tissue we

TABLE 1

Pseudopregnant Rats/Means of Results

Day of Pseudopregnancy		0	1	2	3	4	5
Body Weight	g +/-	173 3	169 5	189 3	184 3	185 9	179 6
Uterine Weight	mg +/-	325 27	275 39	255 8	266 6	295 7	259 21
Thymic Weight	mg +/-	292 14	306 125	273 18	262 15	275 22	270 8
Cell content x10^6	+/-	893 53	962 125	815 65	930 69	954 133	902 35
Cell density x 10^6/100 mg tissue	+/-	308 22	320 20	297 9	329 16	338 21	333 9
Labelled cells	% +/-	8.23 1.14	7.04 0.47	8.96 0.87	12.82 0.85	8.88 1.16	5.19 1.28
URLN Weight	mg +/-	22.67 3.63	23.60 2.59	26.50 2.25	28.82 4.46	22.10 3.49	21.28 1.12
Cell content x 10^6	+/-	8.99 2.72	6.25 1.74	13.94 1.76	14.43 2.95	10.22 3.60	7.25 1.14
Cell density x 10^6/100 mg tissue	+/-	35.75 6.15	25.54 4.69	52.65 4.59	50.00 6.32	42.05 6.86	34.31 4.95
Labelled cells	% +/-	1.47 0.29	1.10 0.18	1.31 0.33	1.23 0.20	1.74 0.22	0.74 0.22

Means of results +/- SE; URLN= uterine regional lymph nodes

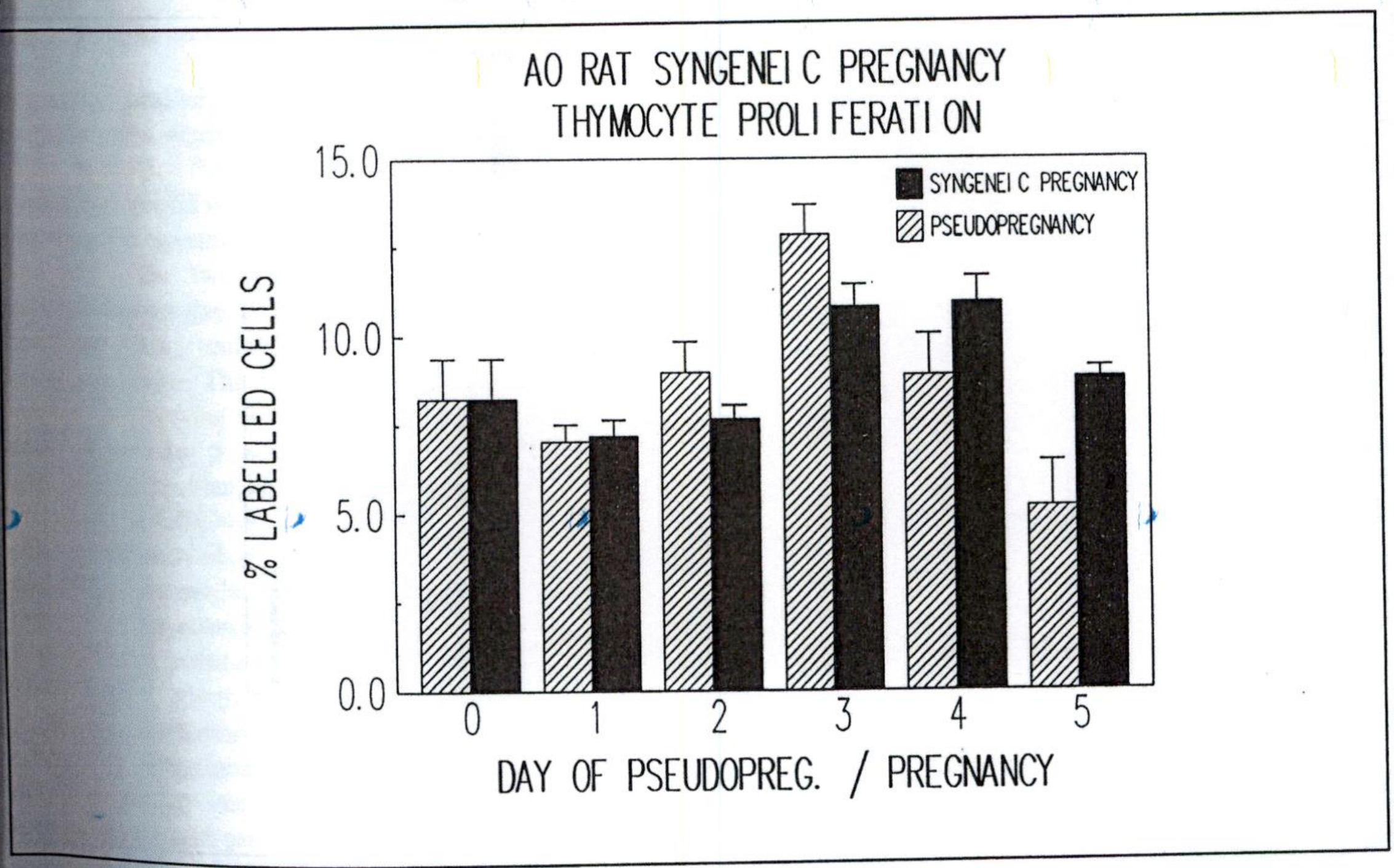

Figure 1. Ao Rat Syngeneic Pregnancy Thymocyte Preoliferation.

developed in Kodak D19 developer, fixed in acid-hardening fixer, stained with hematoxylin and eosin and mounted. A non-random strip counting method was used to assay, at x400 magnification, the number of labelled and unlabelled cells and thus the percentage of cells labelled. Only cells in the DNA-synthesis 'S' phase of mitosis incorporate thymidine (Ford, 1978) and assuming a random distribution of mitotic phases, a constant proportion of the actively proliferating cells, approaching 50% (Wagner et al., 1967) will be in the 'S' phase. The consistency and low standard errors of the results obtained from each group in this study support this assumption. Consequently, it seemed reasonable to use the figures for percentage labelling as a measure of proliferative activity; although not an absolute measure it is an accurate reflection of the relative differences between groups.

Results

A number of observations were made on each lymphoid tissue taken from the females during the estrous cycle, early pseudopregnancy and the pre-implantation phase of syngeneic pregnancy. Pseudopregnancy has a mean of 12 to 13 days (Long and Evans, 1922), while successful gestation is usually completed 21 to 23 days after mating (Mather and Rushmer, 1979). The time interval between observations and the restriction of the observation period to the first 5 days of pseudopregnancy and syngeneic pregnancy, allow results obtained on different days in either state and obtained on comparable days in both states compared. Statistical analysis of data was by the an of variance. Differences between groups were cons to be significant if 'P' was 0.05 or less. The resu shown in tables 1 & 2 in which the numerical val the group means are given with their standard Figures 1 & 2 illustrate the changes that oc radiolabelled cell counts in the thymus and u regional lymph nodes. Although body weigh significantly heavier on day 1 of pregnancy than 1 of pseudopregnancy, no other significant cha body weight was recorded. No significant chan uterine weight were observed during estru pseudopregnancy. In contrast, uterine weigh significantly greater on day 1 of pregnancy than at or on the other days of pregnancy. In addition, on da and 5 the uterine weight was significantly greater pregnancy than pseudopregnancy. No significant c in thymic weight, total thymocyte count or thym density were observed during the first five da pregnancy or pseudopregnancy nor for any day co with the estrous values. There were however sign increases in the total lymphocyte count and thym density on day 2 of pregnancy compared with the day of pseudopregnancy. Thymocyte proliferation

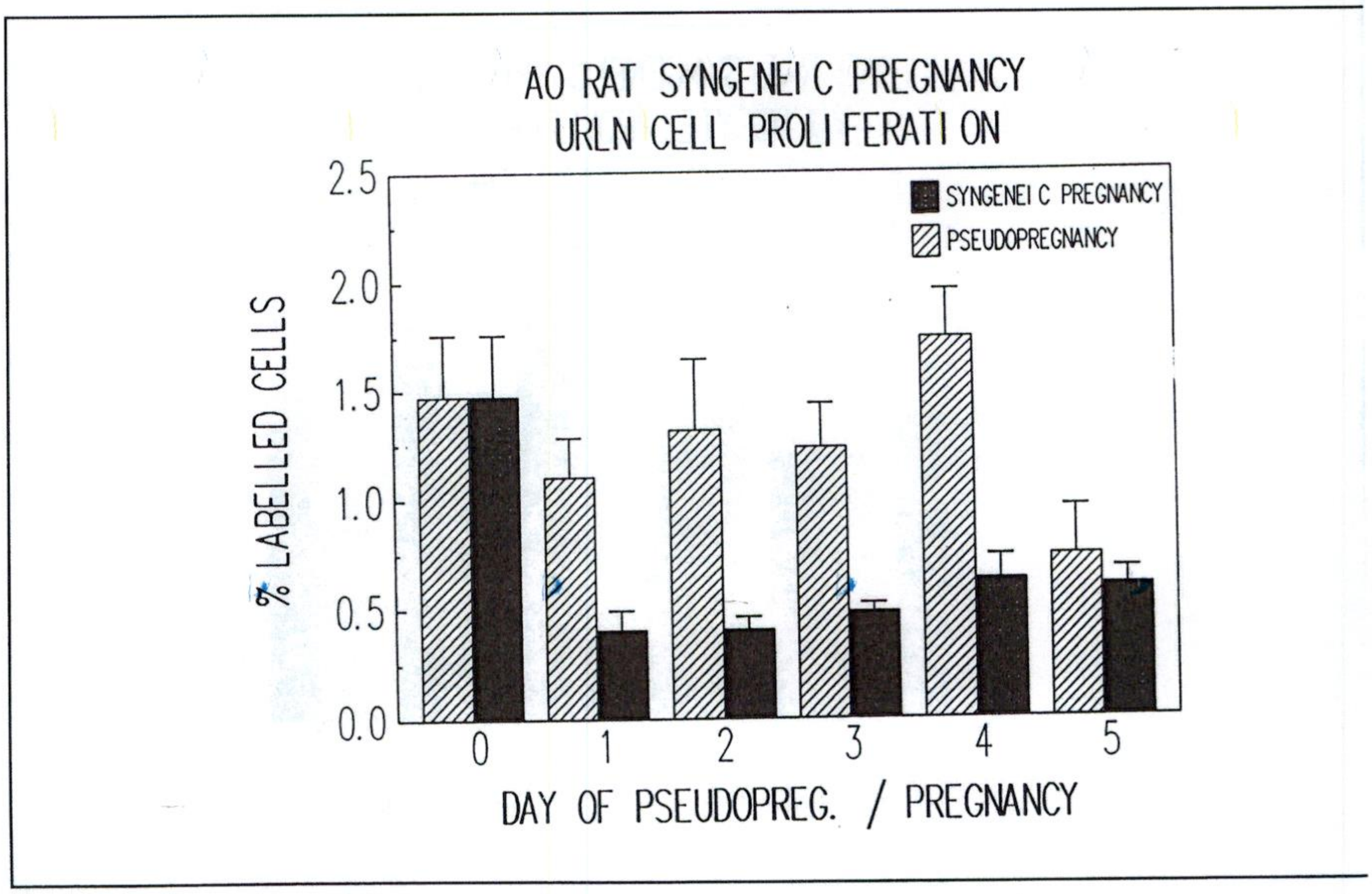

Figure 2. Ao Rat Syngeneic Pregnancy URLN Cell Proliferation.

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TABLE 2

Syngeneically Pregnant Rats/Means of Results

Day of Pseudopregnancy		0	1	2	3	4	5
y Weight	g	173	169	188	189	188	192
	+/-	3	5	4	4	4	4
erine Weight	mg	325	389	322	293	278	326
	+/-	27	28	17	24	19	7
ymic Weight	mg	292	280	288	297	311	290
	+/-	14	21	10	10	16	21
l content x10^6		893	961	1007	941	972	976
	+/-	53	98	64	61	63	99
l density x 10^6/100 mg tissue		308	338	348	315	311	332
	+/-	22	14	15	11	8	14
belled cells	%	8.23	7.18	7.64	10.80	10.92	8.80
	+/-	1.14	0.45	0.40	0.64	0.75	0.31
RLN Weight	mg	22.67	21.35	29.20	25.52	28.42	27.22
	+/-	3.63	1.05	1.68	0.89	2.19	2.34
l content x 10^6		8.99	9.19	25.27	14.83	17.53	18.13
	+/-	2.72	1.07	3.12	1.13	3.41	2.38
l density x 10^6/100 mg tissue		35.75	42.56	85.35	57.94	59.86	67.31
	+/-	6.15	3.50	7.76	3.35	7.97	8.76
belled cells	%	1.47	0.40	0.40	0.48	0.63	0.60
	+/-	0.29	0.09	0.06	0.04	0.11	0.08

ıns of results +/- SE; URLN= uterine regional lymph nodes

gnancy peaked on days 3 and 4 and the values orded were significantly greater than those on days ,2 and 5. Similarly, during pseudopregnancy mocyte proliferation peaked on day 3 and was nificantly greater than on days 0,1,2,4 and 5. mparing the two physiological states, thymocyte liferation on day 3 was significantly greater during udopregnancy and on day 5 was significantly greater ing pregnancy. The only significant observation in the ights of uterine regional lymph nodes (URLN) urred on day 5 when the pregnancy nodes were nificantly heavier than the pseudopregnancy nodes. hough the URLN total cell counts during estrus and udopregnancy showed no significant differences, the al cell count on day 2 of pregnancy was significantly ater than the estrous count and those on the other days pregnancy, while those on days 2,4 and 5 were nificantly greater than on day 0 and day 1 of gnancy. Furthermore, the URLN total cell counts oughout pregnancy were greater than those of udopregnancy and significantly so on days 2 and 5. th pregnant and pseudopregnant URLN cell densities aked on day 2 with all the pregnancy values being eater than those of pseudopregnancy and significantly so on days 1,2 and 5. The level of lymphocyte proliferation in the URLN during estrus and pseudopregnancy showed no significant differences but on each of the five days of pregnancy it was significantly lower than that recorded at estrus. Furthermore, the levels of lymphocyte proliferation in these nodes during pseudopregnancy were greater than those of pregnancy and significantly so on days 1,2,3 and 4.

None of the popliteal lymph node observations made during estrus, pseudopregnancy or pregnancy showed any significant differences.

Discussion

Experimental or coital stimulus of the cervix interrupts the repetitive pattern of ovarian hormone secretion and alters the hormonal status of the female animal. In addition to her altered hormonal status, the post-coital female also has the ejaculate and the newly formed zygotes present within her upper reproductive tract. Any significant differences between the pregnant and pseudopregnant animals during the pre-implantation period might reasonably be assumed to be causally associated with the presence of the ejaculate and the

products of conception. Furthermore, since this experiment involved syngeneic mating, the post-coital female was not exposed to any allogeneic challenge from spermatozoa, or other cellular elements in the ejaculate or the presence of semi-allogeneic embryos. Therefore, any significant differences observed must be the result of exposure to sperm specific antigens, male specific antigens and seminal plasma.

Since this study was designed to assess any in-vivo immunosuppressive action of seminal plasma, the principal observations concerned the lymphoid tissues. However, body weights and uterine weights were also recorded; body weight to determine lymphoid tissue weight per unit body weight, in which no significant changes were observed, and uterine weight because the uterus receives the ejaculate and is the site of implantation. Since body weight did not change significantly from the estrous value during either pregnancy or pseudopregnancy it would be inappropriate to assign any biological significance to the significant increase in body weight on day 1 of pregnancy compared with day 1 of pseudopregnancy. Although uterine weight at estrus and those during pseudopregnancy showed no significant changes it was significantly greater on day 1 of pregnancy compared with estrus and other days of pregnancy, and on days 1,2 and 5 of pregnancy it was significantly heavier than on the comparable days of pseudopregnancy. Since the uterine cavity was opened and washed prior to weighing, the initial differences in weight must reflect changes in the uterus itself in response to the ejaculate while the difference on day 5 must reflect the appropriate preparation of the endometrium for implantation. Thus, exposure of the endometrium to the ejaculate and implanting embryos induced an increase in uterine weight. There is no direct evidence available from this investigation as to the mechanism or mechanisms by which uterine weight was increased but the presumptive causes are increased vascularisation of the uterus in response to the ejaculate together with increased cellular proliferation within the endometrium in readiness for implantation.

The thymus is a primary lymphoid organ in which lymphocyte activity is thought to be largely independent of antigen challenge (Nossal, 1984). The generally held view is that pre-T-lymphocytes enter the thymic cortex from the blood and a small proportion of these cells migrate to the medulla where they acquire their specific immunological functions before being exported to the peripheral lymphoid tissues (Golub and Green, 1991). Although large at birth the thymus reaches its maximum size at puberty after which it begins to atrophy while retaining its function (Calzolari, 1898). Apart from age, the other important factors which affect the size of the thymus are the steroid hormones produced by the adrenals (Boyd, 1932) and gonads (Chiodi, 1940; Leeming et al., 1984). The observations made in this study sugg mating has only minimal effects upon the t Certainly there are significant increases in th thymocyte count and thymic cell density on da pregnancy compared with the same dem pseudopregnancy but this is followed on day pregnancy by a significant depression in the le thymocyte proliferation compared with th pseudopregnancy. Since the same pattern of thy proliferation occurred in both physiological sta suggests that if the ejaculate was responsible increases in total thymocyte count and thymic cell on the second post-coital day, an effective mech perhaps involving the action of seminal plas operating to sustain thymic homeostasis. The sign increase in thymocyte proliferation on day 5 of preg compared with the same day of pseudopregnancy r the result of hormonal secretions by the impl embryos.

It has been long established that exposure of a alloantigens is followed by significant changes draining lymph nodes. After a skin allograft the re lymph nodes increase in size and evidence of lymph proliferation as well as lymphocyte accumulat observed (Scothorne and McGregor, 1955; Parrott & Sousa, 1970; Zatz and Lance, 1971). The recruitm lymphocytes from the circulating pool reflects importance in the initiation of the immune response and Gowan, 1967; Sprent et al., 1971; Rowley 1972). An earlier study of the uterine regional l nodes during the first 10 days of syngeneic and allog rat pregnancy demonstrated that syngeneic mating w effective as allogeneic mating in producing signi increases in the total cell content and cell density URLN when compared with the estrous values, wi the accompanying significant increases in lymph proliferation observed after allogeneic mating (Sha al., 1981). In the present experiment, syngeneic m was followed by significant increases in the tota content on days 2 and 5 of pregnancy compared wi same days of pseudopregnancy and by signi increases in the cell density on days 1,2 and pregnancy compared with the same days pseudopregnancy. The most important observation i experiment, however, was the significant depressi lymphocyte proliferation on all five days of pregn when compared with the estrous value and on days and 4 when compared with the same days pseudopregnancy.

These results indicate that the sperm specific anti and male specific antigens in the ejaculate in lymphocyte accumulation in the URLN while the sem plasma depresses 'in situ' lymphocyte proliferation therefore has an immunosuppressive action ' in vi comparable to that which it demonstrates ' in vitro '.

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