Purine utilisation, de novo synthesis and degradation in mouse preimplantation embryos

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Summary

The importance of de novo purine synthesis as opposed to the reutilisation of metabolites by salvage pathways, and the nature of the excretory product(s) of purine degradation, have been examined in cultured preimplantation mouse embryos. In the presence of azaserine and mycophenolic acid, which inhibit de novo purine synthesis, embryo cleavage was blocked prior to compaction, the precise stages at which this occurred depended on whether the cultures were established on day 1 or day 2 after fertilisation, and indicated that salvage pathways were insufficient to fulfil the demand for nucleotides during early preimplantation development. The end-product of purine degradation appeared to be xanthine, which was excreted in very small amounts on days 1, 2 and 3, with a pronounced rise from the early to late blastocyst. Uric acid formation or excretion could not be detected. Exogenous hypoxanthine and adenine, which partially inhibited development, were taken up by the embryos and converted to xanthine, most probably by salvage pathways, since the enzyme xanthine oxidase, which converts hypoxanthine directly to xanthine and then to uric acid, could not be detected. Exogenous guanine had little effect on development and was also converted to xanthine, but in this case, the conversion was probably in a single step, via the enzyme guanase.

Key words: purines, xanthine, hypoxanthine, azaserine, preimplantation embryo, development, mouse, HPLC.

Introduction

Mouse preimplantation embryos may be grown in chemically defined media consisting of ions, simple energy sources and a macromolecule such as albumin (Pratt, 1987). Although DNA and RNA are synthesized during the preimplantation period, the addition of precursors of nucleic acid synthesis to the incubation media is not required (Whitten, 1971; Brinster, 1973).

Purine nucleotides are synthesized in adult mice as in most other mammals either by de novo biosynthesis or by the "salvage" pathway, whereby reutilisation of purines derived from nucleotide degradation occurs (Murray, 1971; Rodwell, 1988).

The purines adenine and guanine can be salvaged by the action of the enzymes adenine phosphoribosyl transferase (APRT) and hypoxanthine-guanine phosphoribosyl transferase (HGPRT), respectively (Fig. 1). Alternatively, they may be broken down, in rodents, to allantoin (Henderson and Paterson, 1973; Hurst, 1980). In humans, the pathway stops with uric acid production, presumably due to the loss, with evolution, of uricase activity.

Radiolabelled purine bases, which are incorporated into nucleotides via the salvage pathway, have been used to study the qualitative and quantitative pattern of nucleic acid synthesis in early mouse embryos (Minz, 1964; Monesi and Salfi, 1967; Epstein and Daentl, 1971; Clegg and Piko, 1982), together with the timing of embryonic genome expression (Young et al., 1978; Flach et al., 1982; Bolton et al., 1984; Schultz, 1986). Data from the incorporation of radiolabelled precursors have shown a continually increased synthesis of new RNA molecules of all major classes, beginning at the 2-cell stage, at which time the embryonic genome is switched on. However, little is known about purine turnover (i.e., synthesis and degradation), and excretion during the first five days of mouse embryo development.

Thompson Ten Broeck (1968) observed increasing inhibition of mouse preimplantation development by adenine, adenosine and guanosine as their concentrations were raised from $10^{-6}$ M to $10^{-2}$ M. Epstein et al., (1971) reported studies on the uptake and utilisation of exogenous guanine and adenine by the mouse preimplantation embryo. There was a major increase in the uptake of radiolabelled guanine between the 2- and 8-cell stages with maximum uptake at the early blastocyst stage; it was also shown that guanine entering the embryo was rapidly salvaged to GMP, which was further converted to GDP and GTP.

The aim of the present study was to assess the importance of the de novo and salvage pathways to preimplantation mouse embryo development, and to
determine the nature of the end-products of purine degradation. Two potent inhibitors of nucleotide synthesis, azaserine and mycophenolic acid, have been used to inhibit the de novo pathway. It has been known since the mid 1950's that azaserine, acting as a competitive antagonist of glutamine, inhibits de novo purine synthesis in mammals and avians (Skipper et al., 1954; Levenberg et al., 1957; Moore and Le Page, 1957). There is evidence that azaserine reacts with the enzyme converting formylglycinamide ribotide (FGAR) to formylglycinamidine ribotide (FGAM). Mycophenolic acid blocks nucleotide synthesis largely by inhibiting the enzyme IMP dehydrogenase, which is involved in the formation of GMP (guanosine monophosphate) from IMP (inosine monophosphate) (Franklin and Cook, 1969).

The approach to the problem of detecting excretion products derived from purine catabolism has been to analyse the culture medium in which embryos were grown. Embryonic development and end-product excretion have been assessed in response to suppletion of the culture medium with hypoxanthine, adenine, guanine, xanthine and uric acid.

High performance liquid chromatography (HPLC) has been used to detect the purine bases, nucleosides and nucleotides. This technique has been successfully applied to the analysis of these compounds in various biological fluids and tissues, such as urine, leukocytes, erythrocytes, liver, brain (Harkness et al., 1983; Wynants and VanBelle, 1985; Brown et al., 1982), oocytes and cumulus cells (Eppig et al., 1985; Downs et al., 1986), and recently, human preimplantation embryos (Leese et al., 1991).

**Materials and methods**

*Embryo collection and culture*

5-8 week old female mice of the inbred strain CBA/Ca × C57BL/6, were superovulated with 5 i.u. PMS (Folligon, Intervet, Cambridge, UK), between 12:00 and 13:00 hours, followed 48 hours later by 5 i.u. HCG (Chorulon, Intervet, UK). After the second injection they were placed with fertile males of the same strain. Mating was checked the following morning by the presence of a vaginal plug (Day1). The cumulus oophorus from embryos recovered from the oviduct on day 1 (20-24 hours following HCG) was dispersed by treatment for 5-10 minutes with 1 mg/ml hyaluronidase (Sigma Chemical Co, Poole, Dorset, UK) in medium M2 (Whittingham, 1971). Subsequent embryonic stages were flushed from the oviduct or uterus, washed in M2 and grown in 2-5 μl droplets of M16 under 3 ml paraffin oil in Petri dishes. The embryos were cultured in groups of 30-120 at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. They were transferred to a new droplet each day between 9:00-12:00a.m. The time interval between the samples was, therefore, approximately 24 hours. The spent-medium drops were either analysed immediately or stored in a 5 μl microcap (Drummond Scientific, UK) at 20°C.

The compounds to be tested (all from Sigma) were added to the culture medium at the following concentrations: 0.05 and 0.1 mM hypoxanthine, 0.05 mM guanine, 0.05 mM adenine, 0.05 mM xanthine, 0.05 mM uric acid and 0.1 mM uric acid. The response of the embryos to azaserine was tested at concentrations of 10, 25, 50 and 100 μg/ml in M16. The extent of inhibition was not significantly different at each of these
between 285 and 300 mosmol/L. For each series of experiments, the osmolarity of the controls and the test media did not differ by more than 5 mOSM. Control cultures containing M16 alone or M16 plus 0.2 M NaOH at a final concentration of 25 µg/ml mycophenolic acid was chosen as appropriate. Each of the compounds was dissolved in 0.2 M NaOH (except for guanine which was dissolved in 0.5 M KOH), and then added in the smallest possible volume to M16 in order to minimise any change in pH or osmolarity. In all cases, the media were adjusted to maintain a pH of 7.2-7.4 and an osmolarity between 285 and 300 mosmol/L. For each series of experiments the osmolarity of the controls and the test media did not differ by more than 5 mOSM. Control cultures containing M16 alone or M16 plus 0.2 M NaOH at a final concentration similar to that used for test substrates were always included.

Purines and metabolic inhibitors were added either on day 1 or day 2. In each case, cultures were continued to the blastocyst stage on day 5.

**HPLC analysis**

HPLC analysis was carried out on a Kontron 400 series system (Kontron Instruments, Watford, Herts, UK) which consisted of a solvent pump, an autosampler injector, a variable wavelength UV detector and a computer data system MSDOS to record the analyses and determine the retention time and peak area (in mV) of the compounds eluted.

The chromatography was performed in an isocratic mode, with a reverse-phase Hypersil, 5 ODS (25 cm x 2 mm) column (Shandon, Runcorn, Chesire, UK) at room temperature. For all the reference samples, the injected volume was 20 µl; for the culture medium samples, it was 14-18 µl.

The mobile phase was 25 mM NH₄H₂PO₄, pH 4.5, prepared with double distilled water and filtered through a 0.4 µm membrane filter immediately before use. The column was cleaned with Analar methanol after approximately 50 samples. Elution was performed at a flow rate of 0.3 ml/min, with the detector set at 254 nm and an absorbance sensitivity of 0.002 AFS.

The peaks corresponding to each compound were identified by comparing the retention times with those of freshly prepared standards. Identification could be confirmed by setting the UV detector to various wavelengths including the one representing the highest absorption of a standard of the pure compound. Further confirmation was obtained by the addition of a known amount of the substance to be identified.

**Analyses of results**

The results were analysed using Student’s t-test and Analysis of Variance (one-way).

**Results**

**Inhibitors of de novo synthesis**

Metabolic inhibitors were added to the incubation medium in an attempt to discover whether embryos could develop to the blastocyst stage when de novo purine synthesis was suppressed.

Of embryos treated with azaserine from day 1, 55% were blocked at the 1-cell stage, while the remainder cleaved successfully to the 2-cell stage, after which division ceased (Table 1). When the embryos were incubated with azaserine from the 2-cell stage (Table 1), 90% of them completed the second cleavage division but did not develop further. Of those embryos that reached the 4-cell stage, many attempted to compact even though they were unable to divide further. Although most embryos were degenerate, some had formed a blastocoel cavity by late day 4.

Hypoxanthine, an activator of the salvage pathway, was added to the cultures together with azaserine, to discover whether the inhibitory effect of the latter was reversible. The concentration of hypoxanthine used (0.1 mM) was high enough to stimulate the salvage pathway (Epstein, 1970; Monk, 1987). There was no inhibition of HGPRT activity at this concentration of hypoxanthine (data not shown). In the presence of hypoxanthine, the proportion of embryos, either those cultured from day 1 or day 2, that completed one division was lower than that in the presence of azaserine alone (see Table 1).

In order to discover whether the inhibitory effects were reversible, the embryos were incubated in azaserine for 24 hours and then transferred to M16 or M16 supplemented with hypoxanthine; no improvement in development was observed.

When the embryos were incubated for 4 hours only in azaserine, there was some stage-dependent reversal of inhibition after transfer to M16 or M16+hypoxanthine;

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**Table 1. Effect of azaserine (50 µg/ml) and azaserine (50 µg/ml)+hypoxanthine (0.1 mM), on mouse embryo development from the 1-cell and the 2-cell stage to the blastocyst**

<table>
<thead>
<tr>
<th>Development</th>
<th>% Development</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>1c*</td>
</tr>
<tr>
<td>2-cell stage</td>
<td>100</td>
</tr>
<tr>
<td>4-cell stage</td>
<td>94.7±13.1</td>
</tr>
<tr>
<td>8- to 16-cell stage</td>
<td>87.4±4.6</td>
</tr>
<tr>
<td>blastocyst</td>
<td>90.9±6.1</td>
</tr>
</tbody>
</table>
| Total no. of embryos | 100          | 143           | 213                      | 59                       | 88

* mean±S.E.M. of 4 independent experiments, † mean±S.E.M. of 6 independent experiments.
Table 2. Effect of mycophenolic acid (25 μg/ml) on mouse embryo development from the 1-, 2- and 4-cell stages to blastocyst

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mycophenolic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1c</td>
<td>2c</td>
</tr>
<tr>
<td>2-cell stage</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>4-cell stage</td>
<td>100</td>
<td>96.1±0.5</td>
</tr>
<tr>
<td>8-cell stage</td>
<td>91.9±2.3</td>
<td>94.8±0.4</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>75.6±3.4</td>
<td>84.3±6.3</td>
</tr>
<tr>
<td>Total no. of embryos</td>
<td>77</td>
<td>117</td>
</tr>
</tbody>
</table>

lc, 2c, 4c refer to cultures from the 1-cell (day 1), 2-cell (day 2) and 4-cell (late day 2, 54-56 hours post HCG) stages, respectively. Values for cultures from the 1- and 2-cell stage are mean±S.E.M. of 3 determinations. Values for cultures from the 4-cell stage are mean of 2 determinations.

when the inhibitor was added at the 2-cell stage, almost 85% of the embryos developed to blastocysts in M16 and 65% in M16+hypoxanthine; reversal of inhibition was reduced when azaserine was added at the 1-cell stage, where some embryos progressed beyond the 4-cell stage, but less than 5% formed blastocysts after transfer to M16 or M16+hypoxanthine. After incubation with the inhibitor for 4 hours at the 8-cell stage, followed by transfer to M16 or M16+hypoxanthine, all the embryos formed blastocysts by day 5; however, these blastocysts had fewer cells than those formed in control cultures without azaserine treatment; 79 ±5 cells and 101 ±10 cells, respectively.

Mycophenolic acid had an inhibitory effect different from that of azaserine. The results, reported in Table 2, showed that the embryos developed to the 8-cell stage after continuous exposure to the inhibitor from day 2. However, when treated with the drug early after fertilisation, (day 1), the number of embryos reaching the 8-cell stage was reduced, and the main feature of these cultures was the inhibition of compaction.

Excretion products

Incubation media were removed after each day of development and assayed for the release of potential excretory products of purine metabolism.

Xanthine was consistently present each day; the amounts excreted were very low, in the range of 0.03-0.1 pmol embryo⁻¹ 24 hours⁻¹ (Fig. 2). There was a small but statistically insignificant fall in xanthine excretion between day 2 and day 3, but a marked increase (P < 0.001) from the early to the late blastocyst stages.

Uric acid, an anticipated end-product, was not detected at any stage. In view of this, its immediate metabolic precursor, xanthine, was added to the culture medium at concentrations of 0.05 and 0.1 mM. Xanthine had no effect on embryo development and its concentration in the medium was unchanged. No uric acid production was detected. The addition of uric acid similarly had no effect on embryo development, and its concentration in the culture medium also remained unchanged.

Addition of purines

(i) Hypoxanthine: exogenous hypoxanthine, added to cultures on either day 1 or day 2, disappeared steadily from the medium (Fig. 3). The hypoxanthine uptake was accompanied by the appearance of xanthine in the medium. The excretion pattern showed a small increase from the 1-cell stage to the compacted morula, with a dramatic rise following cavitation (Fig. 2). When 1-cell embryos were exposed to 0.05 mM hypoxanthine, the
proportion developing to blastocysts was significantly lower ($P < 0.05$) than those treated from the 2-cell stage (Table 3). For those established on day 1, 82% of control and 65% of hypoxanthine-treated cultures reached the blastocyst stage, whereas there was no difference for those started on day 2.

(ii) Guanine: 0.05 mM guanine, added at the 1-cell stage, inhibited development to hatched blastocysts by only 10% relative to the controls, and when added at the 2-cell stage, gave no significant inhibition (Table 3). The guanine was rapidly utilised by the embryos, such that after 24 hours, all the purine had been consumed. This was true of all the developmental stages. The amount of xanthine excreted after guanine addition (Fig. 4), was extremely high (0.125 pmol embryo$^{-1}$ hour$^{-1}$ for each stage), with the amount excreted by control embryos less than 1% of this value. The total amount of guanine consumed was 0.183 pmol embryo$^{-1}$ hour$^{-1}$, thus, 70% could be accounted for by xanthine appearance in the medium. This implied that only 30% was converted to guanine nucleotides by the salvage pathway.

(iii) Adenine: when embryos were exposed to 0.05 mM adenine from the 1-cell stage, their development to blastocysts was inhibited by almost 55% (Table 3). The pattern of xanthine excretion in this case was qualitatively similar to that of hypoxanthine but the values were lower; 0.09±0.0125 pmol embryo$^{-1}$ 24 hour$^{-1}$ for the developmental stages before cavitation, and 0.47±0.05 pmol embryo$^{-1}$ at the blastocyst stage (Fig. 2). The increase in xanthine excretion was not due to degenerate or arrested embryos. This was shown by flushing early blastocysts from the uterus, with M16, on day 4 and culturing them in the presence of adenine for 24 hours. These embryos developed to expanded blastocysts excreting xanthine in similar amounts to those cultured continuously from day 1.

Discussion

The main conclusions of this study are that the preimplantation mouse embryo is dependent on de novo synthesis for its supply of purine precursors required for nucleic acid formation, and that xanthine appears to be the end-product of purine degradation.

Table 3. The effect of supplementation with hypoxanthine, adenine and guanine (all 0.05 mM) on the development of 1-cell and 2-cell mouse embryos to blastocyst

<table>
<thead>
<tr>
<th></th>
<th>% Development</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>2-cell stage</td>
<td>1c*</td>
</tr>
<tr>
<td>1-cell stage</td>
<td>100</td>
</tr>
<tr>
<td>2-cell stage</td>
<td>100</td>
</tr>
<tr>
<td>8- to 16-cell stage</td>
<td>98.7±1</td>
</tr>
<tr>
<td>blastocyst</td>
<td>84.9±1.2</td>
</tr>
<tr>
<td>Total no. of embryos</td>
<td>89</td>
</tr>
</tbody>
</table>

1c, 2c refer to cultures from the 1-cell and 2-cell stages, respectively.
* mean±S.E.M. of 3 independent experiments.
† mean±S.E.M. of 4 independent experiments.
Values for adenine (2-cell) are the mean of 2 independent experiments.
The importance of de novo synthesis and the salvage pathway were compared by the use of inhibitors. Azaserine, which inhibits nucleotide and therefore DNA synthesis, might have been expected to inhibit cleavage completely. That the embryos were able to complete one division in the presence of the inhibitor may be due to the availability of sufficient intracellular pools of nucleotides and/or their precursors (particularly those of guanine), which are possibly maternally derived, or formed shortly after fertilisation. Another possible explanation is that the embryos were exposed to the inhibitor late in the cell cycle, when DNA replication had started, and sufficient RNA molecules had been synthesized, such that a reduction in the availability of precursors did not impair cell division. The latter hypothesis is supported by the finding that embryos incubated in azaserine at approximately 21 hours post HCG did show a restricted ability for division compared to those exposed three hours later.

In experiments such as these it is difficult to distinguish true inhibition of a biosynthetic pathway from possible non-specific toxic effects. In addition, inhibitors may affect more than one metabolic pathway. For example, it is possible that azaserine could exert its inhibitory effect on glutamine uptake or metabolism as opposed to glutamine incorporation into purines (Hundal et al., 1990). We consider this unlikely. While glutamine is beneficial to mouse preimplantation embryo development, and can help overcome the "2-cell block" in embryos from random-bred mice (Chatot et al., 1990), it has to be added to the culture medium, and in none of the present studies was this the case. Furthermore, during the early stages of development, when azaserine was added, utilisation of glutamine by mouse embryos is low (Gardner et al., 1989).

One approach to the problem of non-specific inhibition is to expose the embryos for a shorter period (4 instead of 24 hours), and to attempt to reverse the inhibitory effect. When this was done, there was partial reversal of the effects of azaserine, following exposure of the embryos to M16 or M16 + hypoxanthine. This suggested that azaserine had not caused embryo degeneration. It was also shown that supplementation of M16 with hypoxanthine did not enhance the reversibility of inhibition by M16 alone. Since hypoxanthine, added to the cultures treated continuously with azaserine, was unable to reverse the effect of the inhibitor, this further supports the proposition that stimulation of the salvage pathway on its own is not sufficient to fulfil the demand for nucleotides.

The suggestion was made earlier, that intracellular pools of nucleic acid precursors may be sufficient to sustain cell division even in the presence of inhibitors. Support for this possibility is provided by the results of the experiments with mycophenolic acid (Table 2), which showed that although the conversion of IMP to GMP was blocked by the inhibitor, the embryos could complete at least two divisions. Hoshii et al. (1988) similarly showed that the inhibitory effect of mycophenolic acid on embryo development could be reversed by guanine, and by xanthine, at a concentration as low as 25 µg/ml. This suggested that xanthine could be salvaged by the activity of HGPRT or XPRT (xanthine phosphoribosyl transferase), an enzyme known to be present in E. coli able to convert xanthine to xanthosine monophosphate (XMP), (Miller et al., 1972). The XMP could then be converted to GMP overcoming the inhibitory effect of mycophenolic acid.

An attempt was made to test this possibility by incubating mouse embryos in a reaction mixture similar to that used for HGPRT determination (Leese et al., 1991), containing xanthine instead of hypoxanthine as substrate. However, the results were negative in that no XMP and/or GMP production was detected.

The appearance of xanthine in the culture medium suggested that this oxypurine is the end-product of purine catabolism in early mouse embryos. Xanthine is formed from hypoxanthine by the enzyme xanthine oxidase (EC 1.2.3.2). The same enzyme converts xanthine to uric acid (Fig. 1). Thus the absence of uric acid in the excretory products is intriguing. It is possible that uric acid is produced, but not at a level high enough to be detected. This proposition was tested by adding xanthine to the culture medium. Neither the morphological appearance of the embryos nor their pattern of excretory products changed as a result of xanthine addition and no uric acid was detected. Uric acid added to groups of embryos was neither taken up nor broken down. Furthermore, there was no uric acid production when extracts of embryos were incubated with xanthine or hypoxanthine as substrate (data not shown). On the other hand, increased amounts of xanthine were produced when the embryos were incubated with hypoxanthine, suggesting that xanthine oxidase was active during the preimplantation stages.

The xanthine could however, have been derived by an alternative route involving guanine deamination (Fig. 1), in a reaction catalysed by guanase (EC 3.5.4.3). This enzyme is very active during the early preimplantation stages in the mouse, before declining in the blastocyst (Epstein et al., 1971). In support of this proposition was the finding that guanine added to the cultures disappeared rapidly and could be accounted for stoichiometrically by the appearance of xanthine in the medium. In other words, exogenous hypoxanthine could be salvaged to form purine nucleotides which gave rise to xanthine indirectly rather than via xanthine oxidase.

Exposure to hypoxanthine at the 1-cell stage, blocked subsequent cell division (Table 3), a finding reported in other strains of mice (Nureddin et al., 1990). Hypoxanthine has also been implicated in causing a 2-cell block in random-bred mouse embryos (Loutradis et al., 1987), and in the maintenance of meiotic arrest in mouse oocytes (Epig et al., 1985; Downs et al., 1986).

Nureddin (1990) and his colleagues had suggested that hypoxanthine is involved in the inhibition of a cell process occurring during the first 30 hours of development, which does not involve the conversion of IMP to AMP or GMP via the salvage pathway. The data (Table 3) showing that the embryos develop as well as the controls, if not better, in the presence of hypoxanthine
added after the 2-cell stage, is consistent with this suggestion. The mechanism by which hypoxanthine blocks the first cleavage division remains obscure.

Adenine, when added at the 1-cell stage, also exerted an inhibitory effect on embryo development, in agreement with the report by Thomson Ten Broeck (1968), showing a concentration-dependent inhibitory effect of a variety of nucleosides and nucleoside bases, including adenine, on mouse preimplantation development. When adenine was administered from day 2, almost all the embryos reached the hatching blastocyst stage (Table 3).

The similarity in the results of hypoxanthine and adenine addition provides further evidence in favour of the proposition that these two purines may be utilised in the salvage pathway, and that the xanthine produced is via nucleotide formation rather than direct conversion from hypoxanthine. The lower xanthine production with adenine could be attributed to lower levels of APRT in comparison to that of HPRT (Epstein, 1970; Monk, 1987; Alexiou et al., 1990).

The metabolic fate of guanine appeared to differ from that of adenine and hypoxanthine. Mouse embryos took up and converted exogenous guanine predominantly to xanthine. Furthermore, the embryos were tolerant of concentrations of guanine, that, in the case of hypoxanthine and adenine, were inhibitory to development (Table 4). The preference for converting guanine directly to xanthine, instead of reutilising it via the salvage pathway was possibly due to the very high activity of guanosine deaminase (guanase).

Why preimplantation embryos should apparently lack xanthine oxidase activity is unclear. This enzyme, with hypoxanthine as a substrate, is used experimentally to generate free radicals in a wide variety of biological systems (Parks and Jacobson, 1987; Friedl et al., 1989). It has been suggested that such oxygen species exert a detrimental effect on early embryo development (Loutradis et al., 1987; Nasr-El-safahani et al., 1990; Legge and Sellens, 1991) and the capacity to form them via xanthine oxidase is something which may not be advantageous to the early embryo.

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